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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application of:	Palsson, Bernhard	Confirmation No.:	1729
Serial No.:	09/923,870	Art Unit:	1631
Filed:	August 06, 2001	Examiner:	Negin, Russell Scott
For:	METHODS FOR IDENTIFYING DRUG TARGETS BASED ON GENOMIC SEQUENCE DATA	Attorney Docket No.: CAM:	12956-003-999 871943-999003

APPEAL BRIEF

Mail Stop Appeal Brief
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This Appeal Brief is submitted in support of the Notice of Appeal filed May 17, 2010, wherein Appellant appeals from the Primary Examiner's rejection of claims 49-52, 56-60, 64 and 68-83.

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REAL PARTY IN INTEREST

The real parties in interest are Bernhard O. Palsson and The Regents of the University of California, who are joint-owners of the rights and interest to this application.

RELATED APPEALS AND INTERFERENCES

Appellant is unaware of any related Appeal or Interference.

STATUS OF CLAIMS

1. Claims canceled: 1-48, 53-55, 61-63 and 65-67.

2. Claims withdrawn from consideration, but not canceled: None.

3. Claims pending: 49-52, 56-60, 64, 68-83.

4. Claims allowed: None.

5. Claims rejected: 49-52, 56-60, 64, 68-83.

6. Claims on appeal: 49-52, 56-60, 64, 68-83.

STATUS OF AMENDMENTS

No amendment has been filed subsequent to the Final Office Action mailed on May 10, 2010.

SUMMARY OF CLAIMED SUBJECT MATTER

The following support for all claims is cited on the page and lines of the specification filed on August 6, 2001.

Independent claim 49 is directed to a method performed in a computer of simulating a metabolic capability of an *in silico* strain of a microbe. The method of claim 49 comprises obtaining a plurality of DNA sequences comprising most metabolic genes in a genome of the microbe to produce an *in silico* representation of a microbe; determining open reading frames of genes of unknown function in the microbe in the plurality of DNA sequences; assigning a potential function to proteins encoded by the open reading by determining the homology of the open reading frames to gene sequences encoding proteins of known function in a different organism; determining which of the open reading frames potentially correspond to metabolic genes by determining if the assigned function of the proteins is involved in cellular metabolism; determining substrates, products and stoichiometry of the reaction for each of the gene products of the metabolic genes having an assigned potential function; producing a genome specific stoichiometric matrix of the microbe produced by incorporating the substrates, products and stoichiometry into a stoichiometric matrix; determining a metabolic demand corresponding to a biomass composition of the microbe; calculating uptake rates of metabolites of the microbe; combining the metabolic demands and the uptake rates with the stoichiometric matrix to produce an *in silico* representation of the microbe; incorporating a general linear programming problem to produce an *in silico* strain of the microbe; performing a flux balance analysis on the *in silico* strain, and providing a visual output to a user of the analysis that simulates a metabolic capability of the strain predictive of the microbe's phenotype Claim 49 is supported throughout the specification as originally filed, for example, by claim 1, by Figures 1 and 2, and at page 3, line 28 through page 4, line 2; page 4, lines 24-25; page 5, lines 9-11 and lines 15-17; page 6, line 1 through page 9, line 17; page 12, line 27 to page 13, line 20; page 13, line 25 through page 14, line 30; page 16, lines 4-6; page 16, line 8 through page 17, line 29; and page 17, lines 19-27.

Independent claim 57 is directed to a method performed in a computer for simulating a metabolic capability of an in silico strain of a microbe. The method of claim 57 comprises providing a nucleotide sequence of a potential metabolic gene in the microbe; determining substrates, products and stoichiometry of the reaction for the gene product of the potential metabolic gene, wherein the gene product having an unknown function in the microbe is assigned a potential function by determining

homology of the nucleotide sequence to gene sequences encoding gene products of known function in a different organism; repeating steps a) and b) for most potential metabolic genes of the microbe to produce an *in silico* representation; producing a genome specific stoichiometric matrix produced by incorporating the substrates, products and stoichiometry of the potential metabolic gene products in the microbe into a stoichiometric matrix; determining a metabolic demand corresponding to a biomass composition of the microbe; calculating uptake rates of metabolites of the microbe; combining the metabolic demands and the uptake rates with the stoichiometric matrix to produce an *in silico* representation of the microbe; incorporating a general linear programming problem to produce an *in silico* strain of the microbe; performing a flux balance analysis on the *in silico* strain; and providing a visual output to a user of the analysis that simulates a metabolic capability of the strain predictive of the microbe's phenotype. Claim 57 is supported throughout the specification as originally filed, for example by claim 1, by Figures 1 and 2, and at page 3, line 28 through page 4, line 2; page 4, lines 24-25; page 5, lines 9-11 and lines 15-17; page 6, line 1 through page 9, line 17; page 13, line 25 through page 14, line 30; page 16, lines 4-6; and page 16, line 8 through page 17, line 29.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 49-52, 56-60, and 68-83 are unpatentable under 35 U.S.C. 103(a) over Pramanik et al., *Biotechnol. Bioengineer.* 56:396-421 (1997), in view of Blattner et al., *Science* 277:1453-1469 (1997) and in view of Kunst et al., *Rev. in Microbiol.* 142:905-12 (1991).

2. Whether claim 64 is unpatentable under 35 U.S.C. 103(a) over Pramanik et al., *Biotechnol. Bioengineer.* 56:396-421 (1997), in view of Blattner et al., *Science* 277:1453-1469 (1997), in view of Kunst et al., *Rev. in Microbiol.* 142:905-12 (1991), as applied to claims 49-52 and 56-60, and in further view of Xie et al., *TIBECH* 15:109-113 (1997).

ARGUMENT

Appellant argues together the patentability of claims 49-52, 56-60, 64 and 68-83.

A. The Examiner's Rejections Under 35 U.S.C. 103(a)

In rejecting claims 49-52, 56-60, and 68-83 as unpatentable over Pramanik et al., *Biotechnol. Bioengineer.* 56:396-421 (1997), in view of Blattner et al., *Science* 277:1453-1469 (1997) and in view of Kunst et al., *Rev. in Microbiol.* 142:905-12 (1991), the Examiner has made the following assertions on the record, *inter alia*, in Office Action mailed May 10, 2010, to meet the Examiner's burden of establishing a *prima facie* obviousness rejection.

(1) Regarding claims 49-52, 56-60, and 68-83, the Examiner has taken the position that Pramanik et al. investigate a stoichiometric model of *E. coli* metabolism. Office Action mailed May 10, 2010: page 5. However, the Examiner concedes that Pramanik et al. does not teach obtaining a plurality of DNA sequences comprising most of the metabolic genes in a genome, determining open reading frames of these genes, assigning functions to the proteins encoded by the open reading frames, and determining which of these the open reading frames corresponds to metabolic genes. Office Action mailed May 10, 2010: page 6. The Examiner also concedes that Pramanik et al. do not teach determining open reading frames of genes having an unknown function and assigning a function to their encoded products based on homology to proteins in a different organism. Office Action mailed May 10, 2010: page 6.

(2) The Examiner asserts that the alleged deficiencies of Pramanik et al. with regard to base claims 49 and 57, is cured by Blattner et al. and Kunst et al. Blattner et al. is alleged to describe mapping the complete genome sequence of *E. coli* strain K-12 and assigning function to proteins by determining similarity in proteins of known function. Office Action mailed May 10, 2010: pages 6-7. The Examiner concedes that neither Pramanik et al. or Blattner et al. teach assigning function to genes of unknown function based on homology to proteins in a different organism. Office Action mailed May 10, 2010: page 7. However, Kunst et al. is alleged to describe sequencing of *B. substilis* genome and express an interest in comparing the *B. substilis* and *E. coli* genomes. Office Action mailed May 10, 2010: pages 7 and 8.

(3) The Examiner claims that it would have been *prima facie* obvious to one skilled in the art at the time of the instant invention to modify the stoichiometric model of Pramanik et al. by using the complete genome sequence of Blattner et al. and the genome comparisons of Kunst et al. because (1) metabolism can be further analyzed; (2) the full sequence enables global approaches to understanding biological function and looking at the evolutionary history, and (3) homology comparisons allow for an analysis of evolutionary differences. Office Action mailed May 10, 2010; page 9. The Examiner further concludes that there is a reasonable expectation of success in combining the stoichiometric matrices corresponding to known biological functions in Pramanik et al. and Blattner et al. with the set of sequences of Kunst et al., wherein a subset of sequences has unknown biological function, because the functions and structural stoichiometries of the relations in Pramanik et al. are analogous to the kinetics of homologous organisms as taught in the introduction of Kunst et al. Office Action mailed May 10, 2010: pages 11-12.

In rejecting claim 64 as unpatentable over Pramanik et al., *supra*, Blattner et al., *supra*, and Kunst et al., *supra*, as applied to claims 49-52 and 56-60, and in further view of Xie et al., *TIBECH* 15:109-113 (1997), the Examiner has made the following assertions on the record, *inter alia*, in Office Action mailed May 10, 2010, to meet the Examiner's burden of establishing a *prima facie* obviousness rejection.

(1) Regarding claim 64, the Examiner asserts that Pramanik et al., Blattner et al., and Kunst et al. make obvious a method of simulating a metabolic capability of an in silico strain of a microbe by simulating metabolism within *E. coli*. Office Action mailed May 10, 2010: page 13. However, the Examiner concedes that Pramanik et al., Blattner et al. and Kunst et al. do not teach calculation of uptake rates by measuring depletion of substrate from the growth media. Office Action mailed May 10, 2010; page 13. The Examiner asserts that Xie et al. studies integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells. Office Action mailed May 10, 2010: page 13.

(2) The Examiner concludes that it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the instant invention to modify the metabolism studies of *E. coli* of Pramanik et al., Blattner et al, and Kunst et al. by using the nutrient depletion studies of Xie et al. because stronger media can be designed to enable better growth of cells. Office Action mailed May

10, 2010: page 14. The Examiner further concludes that there would have been a reasonable expectation of success in applying the animal cell study of Xie et al. to the bacterial studies of Pramanik et al. and Blattner et al. because when all cells are cultured, whether animal or bacterial, the cells need nutrients to survive, and as a result, all species of cells deplete their culture media of these nutrients. Office Action mailed May 10, 2010: page 14.

B. Appellant's Rebuttal of the Rejections Under 35 U.S.C. 103(a)

(1) Obviousness:

A claimed invention is unpatentable if the differences between it and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the pertinent art. 35 U.S.C. § 103(a) (2000); *Graham v. John Deere Co.*, 383 U.S. 1, 13-14 (1966). The U.S. Court of Appeal for the Federal Circuit recently re-iterated the proper standards for making determinations under § 103. *In re Kahn*, 441 F.3d 977 (Fed. Cir. 2006). First, the scope and content of the prior art is determined, the differences between the prior art and the claims at issue are ascertained along with the level of ordinary skill in the pertinent art. Against this background, a determination is made whether the subject matter would have been obvious to a person of ordinary skill in the art at the time of the asserted invention. *Id.* at 985 (citing *Dann v. Johnston*, 425 U.S. 219, 226 (1976) and *Graham v. John Deere Co.*, 383 U.S. 1, 13-14 (1966)).

To reject claims in an application under section 103, an examiner must show an unrebutted *prima facie* case of obviousness . . . . On appeal to the Board, an Appellant can overcome a rejection by showing insufficient evidence of *prima facie* obviousness or by rebutting the *prima facie* case with evidence of secondary indicia of nonobviousness. *In re Rouffett*, 149 F.3d 1350, 1355 (Fed. Cir.1998).

The Federal Circuit has further noted that "[m]ost inventions arise from a combination of old elements and each element may often be found in the prior art." *Kahn* at 986. However, mere identification in the prior art of each element is insufficient to defeat the patentability of the combined subject matter as a whole. Rather, a party alleging invalidity due to obviousness must articulate the reasons one of ordinary skill in the art would have been motivated to select the references and to combine them to render the claimed invention obvious. *Id.* at 986. Additionally, "a reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path

that was taken by the applicant." *In re Gurley*, 27 F.3d 551, 553 (Fed. Cir. 1994); see *KSR Int'l Co. v. Teleflex Inc.* 550 U.S. 398; 127 S. Ct. 1727, 1739–40 (2007) (explaining that when the prior art teaches away from a combination, that combination is more likely to be nonobvious).

The U.S. Patent and Trademark Office recently promulgated guidelines for Examiners in making obviousness determinations in view of the U.S. Supreme Court's decision in *KSR Int'l Co. v. Teleflex Inc.* 550 U.S. 398; 127 S. Ct. 1727; 167 L. Ed. 2d 705, 82 USPQ2d 1385, 1395 (2007) (see MPEP §2141). One important feature of the guidelines is an *explicit requirement* that an Examiner provide articulated reasons for the factual determinations underlying an asserted *prima facie* case of obviousness. This focus is consistent with the rule set down in the *KSR* decision that a factfinder must provide "reasons" why an invention would have been obvious to one of ordinary skill in the art. *KSR* at 1741. In explicating this aspect of the Supreme Court's decision, the guidelines set forth several different rationales that can be used to support an obvious rejection (see MPEP §2143). The guidelines further set forth explicit factual findings that an Examiner must articulate to support an obviousness rejection under each rationale. In the present case the Examiner has applied the "teaching, suggestion or motivation" test, identified in the guidelines as rationale (G). For an obviousness rejection based on this rationale for combining references, the Examiner *is required to articulate* the following: (1) a finding that there was some teaching, suggestion, or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine reference teachings; (2) a finding that there was reasonable expectation of success; and (3) whatever additional findings based on the Graham factual inquiries may be necessary, in view of the facts of the case under consideration, to explain a conclusion of obviousness. While it is proper for the motivation to combine to be implicit and be found in the knowledge of one of ordinary skill in the art, or, in some cases, the nature of the problem to be solved (*KSR Int'l Co. v. Teleflex Inc.*), Appellant respectfully submits that the Examiner has presented insufficient evidence of *prima facie* obviousness. As discussed below, the Examiner has failed to articulate a motivation to modify the stoichiometric model of Pramanik et al. with the genome sequence of Blattner et al. using the comparisons of Kunst et al. to arrive at the claimed methods. Furthermore, the Examiner fails to articulate a finding that there would have been a reasonable expectation of success when combining the cited references (see discussion below).

Still further, the Examiner has ignored secondary indicia of nonobviousness that successfully rebut any *prima facie* case of obviousness, including skepticism of experts and unexpected results.

"Expressions of disbelief by experts constitute strong evidence of nonobviousness." *Environmental Designs, Ltd. v. Union Oil Co. of Cal.*, 713 F.2d 693, 698, 218 USPQ 865, 869 (Fed. Cir. 1983) (citing *United States v. Adams*, 383 U.S. 39, 52, 148 USPQ 479, 483-484 (1966)) (The patented process converted all the sulfur compounds in a certain effluent gas stream to hydrogen sulfide, and thereafter treated the resulting effluent for removal of hydrogen sulfide. Before learning of the patented process, chemical experts, aware of earlier failed efforts to reduce the sulfur content of effluent gas streams, were of the opinion that reducing sulfur compounds to hydrogen sulfide would not adequately solve the problem.). In its discussion of *United States v. Adams*, 383 U.S. 39, 40 (1966), the Court in *KSR* further indicated that the presence of unexpected results supports conclusions that the invention is not obvious to those skilled in the art. *KSR* at 1740.

(2) Appellant's Rebuttal to Rejection of Claims 49-52, 56-60, and 68-83 as Allegedly Unpatentable Over Pramanik et al., *supra*, Blattner et al., *supra*, and Kunst et al., *supra*.

(a) No Motivation to Combine References

Appellant respectfully contends that the Examiner has failed to articulate a *prima facie* case of obviousness, at least in part, by not providing a teaching, suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine reference teachings. In the Office Action mailed May 10, 2010, on page 9, the Examiner appears to suggest that the motivation to combine Pramanik et al. with Blattner et al. "would have been that by knowledge of the full genome of E. coli, not only can metabolism be further analyzed, but also knowledge of the entire sequence of E. coli enables global approaches to understanding biological function in living cells and has led to new ways of looking at the evolutionary history of bacteria [see Blattner et al., first paragraph of introduction on page 1453]." As set forth in the Response filed March 24, 2008: pages 8-9, the latter part of this sentence is almost verbatim from the last sentence of the first paragraph of Blattner et al. Turning to this paragraph in Blattner et al., this passage is set forth in the following context:

> Genome sequences, especially those of well-studied experimental organisms, help to integrate a vast resource of biological knowledge and serve as a guide for further experimentation. Availability of the complete set of genes also enables global approaches to biological function in living cells and has led to new ways of looking at the evolutionary history of bacteria.

Blattner et al., p. 1453, para. 1 (citations omitted).

It is clear that this passage in Blattner et al. relates generally to the knowledge gained from having genome sequence information and its ability to serve as a guide for further experimentation. Such general statements about genome sequences enabling "global approaches to biological function in living cells" and providing "new ways of looking at the evolutionary history of bacteria" fail to provide any motivation to combine Blattner et al. with Pramanik et al. or to modify Pramanik et al. to arrive at the claimed methods.

Furthermore, in the Office Action mailed May 10, 2010, on page 9, the Examiner appears to suggest that the motivation to combine Pramanik et al. with Blattner et al. and Kunst et al. "would have been that such homology comparisons [between *E. coli* and *B. substilis* metabolism genes and proteins] allow for an analysis of the differences in evolution between the two different, but related, organisms to result in improvement in industrial applications [see last sentence on page 905 of Kunst et al. and first paragraph on page 906 of Kunst et al.]." As set forth in the Response filed February 2, 2010: pages 10-11, the Examiner applied Kunst et al. because "the 'unknown' functions of protein sequences are identifiable by homology comparison with corresponding sequences in other organisms." Office Action mailed Dec. 18, 2008, page 8, para. 1 (quotes original). However, the support cited by the Examiner is the passage in Kunst et al. expressly teaches that the majority of genes will not have either (1) a known function or (2) sequence similarity with proteins in other organisms. Therefore, the function of these genes will be unknown. Kunst et al., page 905, para. bridging columns 1 and 2.

Appellant points out that the Examiner provides no reason why one of ordinary skill in the art would arrive at the claimed invention in light of a large number of genes that have no identifiable function or match in another organism. Nor would such a large number of genes with unidentifiable function motivate one to incorporate such information into a model of the invention. Appellant pointed out these deficiencies in the Response filed June 18, 2009: page 10, wherein Appellant stated:

> A teaching that a majority of genes will have an unknown function fails to provide any incentive or motivation for one to combine Pramanik et al. and Blattner et al. with Kunst et al. because it informs the skilled person in the art that there is a high likelihood that incorrect information will be incorporated into the model. Incorrect assignment and incorporation into a stoichiometric model of putative metabolic genes or genes with unknown function as a metabolic gene will lead to inaccurate fluxes and diminution in the ability of the model to correctly simulate or predict a phenotype of the microbial organism. Accordingly, both Blattner et al. and Kunst et al., while they report on sequenced genomes and comparative analysis, teach that one skilled in the art, upon a

> careful reading of Pramanik et al., Blattner et al. and Kunst et al., would not be motivated to combine these references to arrive at the invention as claimed because incorporation of incorrect information leading to a less predictive model is a likely possibility. *Id.* (emphasis added)

Still further, Appellant asserts that Kunst et al. is redundant with Blattner et al. and provides little, if anything, new to the previously withdrawn obviousness rejection based on the combination of Pramanik et al. and Blattner et al. Office Action mailed September 5, 2008. In this regard, the further combination with Kunst et al. fails to improve on the combination of Pramanik et al. and Blattner et al. because Blattner et al. did do sequence comparisons and found that, despite such comparisons, a large fraction of the *E. coli* open reading frames could not be assigned a function based on homology and that a significant majority of the polypeptides encoded by the *E. coli* open reading frames had no match in another organism. As described above, Kunst et al. similarly admits that the majority of genes will have unknown function after sequence analysis. Kunst et al., page 905, para. bridging columns 1 and 2.

Hence, Blattner et al. and Kunst et al. are duplicative and neither would lead one of skill in the art to arrive at the claimed invention when combined with Pramanik et al. because there is insufficient specificity in identifying an encoded protein and accurately assigning a function when it is deduced from genomic sequence information.

Still further, Pramanik et al. provides no incentive or motivation for one of skill in the art to combine Pramanik et al. and Blattner et al. with Kunst et al. to arrive at the claimed methods and, if anything, teaches away from the claimed methods. At best, Pramanik et al. describe construction of a metabolic model using only biochemical data. While Pramanik et al. does list some genes, the list is incomplete compared to the model and consists only of known genes encoding proteins with known biochemical activity. As set forth in the Response filed June 18, 2009: page 9,

> There is no teaching, suggestion or hint of an incentive to use information other than know[n] biochemical data, including gene or genomic information, to modify or expand the content of the model. Rather, Pramanik et al. state "[t]here was close agreement between the predicted and experimentally determined flux values" (page 410, col. 1, para. 3) and "[t]his metabolic model should be a useful tool for studying the effects of reengineering pathways" (page 410, col. 2, para. 2).

Contrary to the claimed methods, Pramanik et al. actually teach away from using models that are not produced from existing biochemical information. As Appellant stated in the Response filed March 24, 2008; page 8, Pramanik et al. is distinguished in the subject application, wherein it describes:

> In one example, Pramanik et al. described a stoichiometric model of *E. coli* metabolism using flux-balance modeling techniques (*Stoichiometric Model of Escherichia coli Metabolism: Incorporation of Growth-Rate Dependent Biomass Composition and Mechanistic Energy Requirements*, *Biotechnology and Bioengineering*, Vol. 56, No. 4, November 20, I 997). However, the analytical methods described by Pramanik, et al. can only be used for situations in which biochemical knowledge exists for the reactions occurring within an organism. Pramanik, et al. produced a metabolic model of metabolism for *E. coli* based on biochemical information rather than genomic data since the metabolic genes and related reactions for *E. coli* had already been well studied and characterized. Thus, this method is inapplicable to determining a metabolic model for organisms for which little or no biochemical information on metabolic enzymes and genes is known. It can be envisioned that in the future the only information may have regarding an emerging pathogen is its genomic sequence.

Application, page 4, first paragraph [emphasis added].

Accordingly, one skilled in the art would conclude that the Pramanik et al. model is successful and that there is no problem to be solved that would benefit by including metabolic reactions deduced from open reading frames of genes of unknown function. Furthermore, Pramanik et al. actually teach away from generating a metabolic model absent actual knowledge of biochemical information. These assertions are corroborated by the Declarations of Dr. Keasling and Dr. Palsson, which were first submitted on June 18, 2009, and resubmitted on February 1, 2010.

Dr. Keasling is the senior author on the cited primary reference, Pramanik et al., and is a leader in metabolic modeling. Referring to the declaration of record by Dr. Keasling, Appellant stated in the Response filed June 18, 2009; page 11:

> Dr. Keasling declares that at the time when the genome sequence of Blattner et al. was available, he did not consider utilizing sequence information to incorporate additional metabolic reactions into his model because the resulting *in silico* model would not have been expected to be predictive of an actual organism's metabolism, ¶7. Dr. Keasling further

> declares that his model, as described in Pramanik et al., would have been expected to produce a large number of inaccurate fluxes and lead to a model that is much less predictable if putative reactions were included based sequence homology comparisons, ¶7. Dr. Keasling also declares that identification and assignment of some open reading frames as putative metabolic enzymes was speculative and likely to result in incorporation of inaccurate information and loss of the resultant model's ability to predict a phenotype, ¶8.

Dr. Palsson is the inventor on the above-identified application and a pioneer in the field of stoichiometric models of metabolism. Referring to the declaration of record by Dr. Palsson, Appellant stated in the Response filed June 18, 2009; page 11:

> Dr. Palsson declares that those skilled in the field of metabolic modeling and engineering would not have been motivated to incorporate the genomic data of Blattner et al. or sequence comparisons of Kunst et al. because the predictability of the resultant model would have been expected to be reduced due to incorporation of incorrect information, ¶¶ 8-9.

In summary, the Examiner has failed to articulate a *prima facie* case of obviousness, at least in part, by not providing a teaching, suggestion or motivation to combine the cited references, either in the references themselves, i.e. the cited portions of the references do not provide an incentive or motivation to combine the cited references and the primary reference actually teaches away from their combination, or in the knowledge generally available to one of ordinary skill in the art, i.e. see the Declarations of Dr. Keasling and Dr. Palsson, which corroborate that there was no motivation, generally known in the art at the time of the invention, to incorporate genomic data of unknown function with a metabolic model using only biochemical data. Appellant maintains that, at least for the reasons articulated above, the disclosure of Pramanik et al., alone or in combination, with Blattner et al. and/or Kunst et al. does not render the claimed methods obvious. Therefore, Appellant respectfully solicits reversal of this rejection.

(b) No Reasonable Expectation of Success

Appellant respectfully contends that the Examiner has also failed to articulate a *prima facie* case of obviousness, at least in part, by not showing that one of skill in the art would have a reasonable expectation of success in practicing the claimed methods based on the cited references. In the Office Action mailed May 10, 2010, on pages 11-12, the Examiner concludes that there is a reasonable

expectation of success in combining the stoichiometric matrices corresponding to known biological functions in Pramanik et al. and Blattner et al. with the set of sequences of Kunst et al., wherein a subset of sequences has unknown biological function, because the functions and structural stoichiometries of the relations in Pramanik et al. are analogous to the kinetics of homologous organisms as taught in the introduction of Kunst et al. Office Action mailed May 10, 2010: pages 11-12. Appellant respectfully points out that the above rationale fails to address the teachings of the cited references in that the majority of genes do not have a homology comparison and that, based on the cited art, one would not have expected incorporation of genes having potential functions to results in a model that was predictive. For example, Blattner et al. teach that 38% of the protein-coding genes have no attributable function (abstract, lines 1-2; page 1458, col. 3, para. 1, lines 1-9, and Table 4) and that nearly 60% of *E. coli* proteins have no match in any other complete genome that was considered (page 1459, col. 2, para. 2, lines 1-3). Kunst et al. also fails to cure the deficiencies or provide any incentive to utilize open reading frames of genes of unknown function in a stoichiometric model because Kunst et al. admits that the majority of genes will have an unknown function.

> At the first level of analysis, the DNA sequence will lead to a complete catalogue of putative protein sequences. These are likely to fall into one of 3 categories: (1) those whose functions are known, (2) those which show similarities with proteins identified in other organisms and which may have similar though not necessarily identical function in *B. subtilis*, and (3) those, probably the majority, whose function is unknown at present.

Id., page 205, para. bridging columns 1 and 2 (emphasis added). As Appellant stated in the Response filed February 1, 2010; page 13:

> [A]bsent a homology comparison for most genes one of ordinary skill in the art could not have had a reasonable expectation of success of incorporating most putative metabolic genes and obtaining a predictive model. Further, identification of some putative metabolic genes was speculative and expected to result in incorporation of inaccurate reactions.

Furthermore, the teachings of Pramanik et al. are contrary to the Examiner's assertion that there is any reasonable expectation of success. As Appellant stated in the Response filed March 24, 2008: page 10:

> Pramanik et al. indicates that "the analytical methods described by Pramanik, et al. can only be used for situations in which biochemical knowledge exists for the reactions occurring within an organism"

> (specification page 4, first paragraph). Thus, one skilled in the art would have had no reasonable expectation of successfully achieving Applicant's claimed methods.

This assertion regarding a lack of reasonable expectation of success, is corroborated by the Declarations of Dr. Keasling and Dr. Palsson, which were first submitted on June 18, 2009, and resubmitted on February 1, 2010. Dr. Keasling declares that incorporating reactions based on homology comparisons of unknown genes with metabolic genes in other organisms was expected to yield inaccurate results because identification and assignment of some open reading frames as a putative metabolic enzyme was speculative and would likely result in some incorrect assignments (see ¶8 of Dr. Keasling's Declaration). Dr. Keasling goes on to declare that incorporation of such inaccurate information was expected to generate wild fluxes if incorporated into a model such as that described in Pramanik et al. (see ¶8 of Dr. Keasling's Declaration). Dr. Palsson declares that it was incomprehensible to those in the field of metabolic modeling and metabolic engineering to incorporate reactions based on putative gene sequence homology without knowledge that the reaction existed in the modeled organism and without any known kinetic or biochemical information because incorrect deductions would decrease the predictive capability of the resulting metabolic model, thus resulting in a loss in the model's accuracy (see ¶9 of Dr. Palsson's Declaration). As Appellant stated in the Response filed June 18, 2009: pages 10-11:

> Drs. Keasling and Palsson declare that one would not have expected the combination to result in a model that is capable of accurately predicting a microbial phenotype.

Additionally, if Dr. Keasling, as the senior author on the cited primary reference, Pramanik et al., did not consider incorporation of genomic information based on, for example, the publication by Blattner et al. or Kunst et al., then it is very unlikely that one of ordinary skill in the art would have considered it as well.

In summary, the Examiner has failed to articulate a *prima facie* case of obviousness, at least in part, by not showing that one of skill in the art would have a reasonable expectation of success in practicing the claimed methods because the analytical methods described by Pramanik et al. can only be used for situations in which biochemical knowledge exists for the reactions occurring within an organism. This assertion is corroborated by the Declaration of Dr. Keasling and Dr. Palsson. Appellant maintains that, at least for the reasons articulated above, the disclosure of Pramanik et al.,

alone or in combination, with Blattner et al. and/or Kunst et al. does not render the claimed methods obvious. Therefore, Appellant respectfully solicits reversal of this rejection.

(c) Secondary Indicia of Nonobviousness:

Although Appellant maintains that the Examiner has not established a *prima facie* case of obviousness, assuming, *arguendo*, that one of ordinary skill in the art were to combine the cited references and one of skill in the art would have a reasonable expectation of success, Appellant maintains that any *prima facie* case has been rebutted by the arguments and/or evidence of record that have established secondary indicia of nonobviousness including skepticism of experts and unexpected results.

(i) Skepticism of Experts

Skepticism of experts in the field of metabolic engineering with regard to the claimed methods has been established by the Declarations of Dr. Palsson and Dr. Nielsen, which were first submitted on June 18, 2009, and resubmitted on February 1, 2010. Dr. Palsson is the inventor on the above-identified application and a pioneer in the field of stoichiometric models of metabolism. As Appellant stated in the Response filed June 18, 2009: page 11, Dr. Palsson declares that Dr. Bailey, a respected and prominent scientist in the art, did not believe that the construction of a metabolic model from genomic sequence information worked as claimed. However, if such model did work as claimed and provided predictable fluxes, the approach represents a very major advance (see ¶11 of Dr. Palsson's Declaration). This disbelief or alternative characterization as a breakthrough discovery is supported in a letter sent from Dr. Bailey to Dr. Palsson (see Exhibit 5 of Dr. Palsson's Declaration) where Dr. Bailey expressly states that the model as claimed is difficult to believe, or alternatively, is a breakthrough in the field (see ¶¶13-16 of Dr. Palsson's Declaration). Dr. Palsson goes on to declare that Dr. Bailey's skepticism was shared by others in the filed at the time of the invention (see ¶15 of Dr. Palsson's Declaration).

The shared skepticism of experts in the field is also established by the Declaration of Dr. Nielsen, a prominent researcher in the field of metabolic models. As Appellant stated in the Response filed June 18, 2009: pages 11-12, Dr. Nielsen declares from personal knowledge that Dr. Bailey publically opposed Dr. Palsson by voicing his belief that it was not possible to predict metabolic functions and cellular physiology using stoichiometric models reconstructed from genomic information

because the large degrees of freedom are likely to yield to false phenotypes (see ¶8 of Dr. Nielsen's Declaration). Dr. Nielsen goes on to declare that the opposition from Dr. Bailey was supported by several others in the public forum, making it "quite clear that there was a general belief that the concept would not work," (see ¶8 of Dr. Nielson's Declaration).

Based on the Declarations and the arguments of record, Appellant maintains that any *prima facie* case of obviousness has been rebutted by the Declarations of Dr. Palsson and Dr. Nielsen, which establish that Dr. Bailey and others shared a general disbelief about the feasibility of constructing a metabolic model from genomic sequence information, which can predict metabolic function and cellular physiology. Appellant maintains that, at least for the reasons articulated above, the disclosure of Pramanik et al., alone or in combination, with Blattner et al. and/or Kunst et al. does not render the claimed methods obvious. Therefore, Appellant respectfully solicits reversal of this rejection.

(ii) Unexpected Results

The unexpected results of the claimed methods have been established by the Declarations of Dr. Keasling and Dr. Palsson, which were first submitted on June 18, 2009 and resubmitted on February 1, 2010. Dr. Keasling is the senior author on the cited primary reference, Pramanik et al., and is a leader in metabolic modeling. Dr. Keasling declares that a model incorporating reactions based primarily on genomic sequence information was "surprising and unexpected" because Dr. Palsson's model did not result in wild fluxes nor decrease in accuracy as compared to the model described in Pramanik et al. (see ¶ 7 of Dr. Keasling's Declaration). Dr. Keasling goes on to declare that it was "surprising" that one could in fact incorporate putative metabolic enzymes into a model such as described in Pramanik et al. and produce results that are predictive of cellular metabolism (see ¶ 8 of Dr. Keasling's Declaration). As Appellant stated in the Response filed June 18, 2009: page 11, in summarizing Dr. Keasling Declaration:

> The ability of a model constructed from genomic sequence data to predict actual cellular metabolism was unexpected (¶¶ 7 and 8). [emphasis added]

Thus, Appellant has demonstrated that even the senior author of the primary reference and a leader in metabolic modeling declares that the results of the claimed methods are unexpected.

The unexpected results obtained from the claimed methods have been further demonstrated by evidence presented with the Declaration of Dr. Palsson. In Exhibit 5 (a letter by Dr. Bailey to Dr. Palsson dated July 8, 1999), Dr. Bailey, a respected and prominent scientist in the art, states:

> This method, if it is correct and if it works, is a very major advance, because it gives a formalism for determining growth rate and pathway rates without any knowledge of any kinetics. I must say it is [a] little hard to believe that this can be done, but maybe you have made a breakthrough. [emphasis added]

As Appellant stated in the Response filed February 1, 2010: pages 14-15, the letter by Dr. Bailey provides direct evidence sufficient for showing that such a result was a breakthrough (showing an unexpected result).

Based on the Declarations and the arguments of record, Appellant maintains that any *prima facie* case of obviousness has been rebutted by the Declarations of Dr. Keasling and Dr. Palsson, which establish the unexpected results obtained from a metabolic model constructed with genomic sequence data, as claimed. Appellant maintains that, at least for the reasons articulated above, the disclosure of Pramanik et al., alone or in combination, with Blattner et al. and/or Kunst et al. does not render the claimed methods obvious. Therefore, Appellant respectfully solicits reversal of this rejection.

(3) Appellant's Rebuttal to Rejection of Claim 64 as Allegedly Unpatentable Over Pramanik et al., *supra*, Blattner et al., *supra*, and Kunst et al., *supra*, as applied to claims 49-52 and 56-60, and in further view of Xie et al., *supra*.

Claim 64 is a dependent claim that incorporates all the elements of base claim 57, as indicated by the Examiner, except for the element of wherein the uptake rates are calculated by measuring the depletion of substrate from growth media of the microbe, the rejection is the same as that of claim 57, which is addressed above. Xie et al. is cited for allegedly describing studies of integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells. As such, Xie et al. does not address any of the deficiencies of the primary references, Pramanik et al., Blattner et al. and Kunst et al. as addressed above. Specifically, Xie et al. does not provide the missing reason to combine Pramanik et al. with Blattner et al. or Kunst et al. or a reasonable expectation of success. Absent a *prima facie* showing of motivation to combine the cited references to arrive at the method of base claim 57, the further combination of references to the additional elements of claim 64, fails to cure the deficiencies for a *prima facie* showing. Furthermore, assuming, *arguendo*, one of ordinary

skill were to combine the cited references, the skepticism of experts and the unexpected results articulated above demonstrates the nonobviousness of the claimed methods, rebutting any allegedly *prima facie* showing. Accordingly, for the reasons articulated in the above remarks with regard to the primary references, Appellant maintains that claim 64 is unobvious over the cited combination of Pramanik et al., Blattner et al., Kunst et al. and Xie et al. Therefore, Appellant respectfully solicits reversal of this rejection.

CONCLUSION

The Examiner did not articulate a *prima facie* basis to deny patentability to the claimed invention under 35 U.S.C. §103(a) for lack of requisite realistic motivation or a reasonable expectation of success. In addition, the Examiner has not give weight to the declaration of Dr. Keasling, Dr. Palsson and Dr. Nielson, which demonstrated the lack of motivation in the art to combine the cited references, a lack of reasonable expectation of success, the skepticism of experts in the field and the unexpected results of the claimed methods. Appellant, therefore, submits that the Examiner's rejection of the appealed claims under 35 U.S.C. §103(a) is not procedurally or legally viable and, hence, solicit reversal thereof.

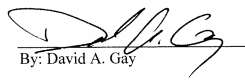
PRAYER FOR RELIEF

For the reasons discussed above, Appellant submits that the examiner's rejections under 35 U.S.C. §103(a) are factually and legally erroneous and, hence, solicit the Honorable Board to reverse the Examiner's rejections of the appealed claims.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due under 37 C.F.R. 1.17 and 41.20, and in connection with the filing of this paper, including extension of time fees, to Jones Day Deposit Account No. 50-3013, referencing our number 871943-999003 and please credit any excess fees to such deposit account.

Date: December 16, 2010

Respectfully submitted,



By: David A. Gay Reg. No. 39,200

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CLAIMS APPENDIX

Claims 1-48 (canceled).

49. A method performed in a computer of simulating a metabolic capability of an *in silico* strain of a microbe, comprising:

obtaining a plurality of DNA sequences comprising most metabolic genes in a genome of the microbe to produce an *in silico* representation of a microbe;

determining open reading frames of genes of unknown function in the microbe in said plurality of DNA sequences;

assigning a potential function to proteins encoded by said open reading by determining the homology of said open reading frames to gene sequences encoding proteins of known function in a different organism;

determining which of said open reading frames potentially correspond to metabolic genes by determining if the assigned function of said proteins is involved in cellular metabolism;

determining substrates, products and stoichiometry of the reaction for each of the gene products of said metabolic genes having an assigned potential function;

producing a genome specific stoichiometric matrix of said microbe produced by incorporating said substrates, products and stoichiometry into a stoichiometric matrix;

determining a metabolic demand corresponding to a biomass composition of said microbe;

calculating uptake rates of metabolites of said microbe;

combining said metabolic demands and said uptake rates with said stoichiometric matrix to produce an *in silico* representation of said microbe;

incorporating a general linear programming problem to produce an *in silico* strain of said microbe;

performing a flux balance analysis on said *in silico* strain, and

providing a visual output to a user of said analysis that simulates a metabolic capability of said strain predictive of said microbe's phenotype.

50. The method of claim 49, wherein said microbe is *Escherichia coli.*

51. The method of claim 49, wherein said genes involved in cellular metabolism comprise genes involved in central metabolism, amino acid metabolism, nucleotide metabolism, fatty acid metabolism, lipid metabolism, vitamin and cofactor biosynthesis, energy and redox generation or carbohydrate assimilation.

52. The method of claim 49, wherein assigning a function comprises performing a homology search using the Basic Local Alignment Search Tool (BLAST).

Claims 53-55 (canceled).

56. The method of claim 49, wherein said uptake rates are calculated by measuring the depletion of substrate from growth media of said microbe.

57. A method performed in a computer for simulating a metabolic capability of an *in silico* strain of a microbe, comprising:

a) providing a nucleotide sequence of a potential metabolic gene in the microbe;

b) determining substrates, products and stoichiometry of the reaction for the gene product of said potential metabolic gene, wherein said gene product having an unknown function in the microbe is assigned a potential function by determining homology of said nucleotide sequence to gene sequences encoding gene products of known function in a different organism;

c) repeating steps a) and b) for most potential metabolic genes of said microbe to produce an *in silico* representation;

d) producing a genome specific stoichiometric matrix produced by incorporating said substrates, products and stoichiometry of the potential metabolic gene products in said microbe into a stoichiometric matrix;

e) determining a metabolic demand corresponding to a biomass composition of said microbe;

f) calculating uptake rates of metabolites of said microbe;

g) combining said metabolic demands and said uptake rates with said stoichiometric matrix to produce an *in silico* representation of said microbe;

h) incorporating a general linear programming problem to produce an *in silico* strain of said microbe;

i) performing a flux balance analysis on said *in silico* strain; and

j) providing a visual output to a user of said analysis that simulates a metabolic capability of said strain predictive of said microbe's phenotype.

58. The method of claim 57, wherein the microbe is *Escherichia coli.*

59. The method of claim 57, wherein said metabolic gene is selected from the group consisting of: genes involved in central metabolism, amino acid metabolism, nucleotide metabolism, fatty acid metabolism, lipid metabolism, vitamin and cofactor biosynthesis, energy and redox generation and carbohydrate assimilation.

60. The method of claim 57, wherein assigning a function comprises performing a homology search using the Basic Local Alignment Search Tool (BLAST).

Claim 61-63 (canceled).

64. The method of claim 57, wherein said uptake rates are calculated by measuring the depletion of substrate from growth media of said microbe.

Claim 65-67 (canceled).

68. The method of claim 51, wherein said genes are involved in central metabolism.

69. The method of claim 51, wherein said genes are involved in amino acid metabolism.

70. The method of claim 51, wherein said genes are involved in nucleotide metabolism.

71. The method of claim 51, wherein said genes are involved in fatty acid metabolism.

72. The method of claim 51, wherein said genes are involved in lipid metabolism.

73. The method of claim 51, wherein said genes are involved in vitamin and cofactor biosynthesis.

74. The method of claim 51, wherein said genes are involved in energy and redox generation.

75. The method of claim 51, wherein said genes are involved in carbohydrate assimilation.

76. The method of claim 59, wherein said genes are involved in central metabolism.

77. The method of claim 59, wherein said genes are involved in amino acid metabolism.

78. The method of claim 59, wherein said genes are involved in nucleotide metabolism.

79. The method of claim 59, wherein said genes are involved in fatty acid metabolism.

80. The method of claim 59, wherein said genes are involved in lipid metabolism.

81. The method of claim 59, wherein said genes are involved in vitamin and cofactor biosynthesis.

82. The method of claim 59, wherein said genes are involved in energy and redox generation.

83. The method of claim 59, wherein said genes are involved in carbohydrate assimilation.

EVIDENCE APPENDIX

Exhibit A – Declaration under 37 C.F.R. §1.132 executed by Dr. Jay D. Keasling with Exhibit 1 attached submitted on June 18, 2009 and February 1, 2010; entered into the record by the Examiner in the Office Action mailed October 1, 2009: pages 15-16.

Exhibit B – Declaration under 37 C.F.R. §1.132 executed by Dr. Bernhard O. Palsson with Exhibits 1-10 attached submitted on June 18, 2009 and February 1, 2010; entered into the record by the Examiner in the Office Action mailed October 1, 2009: pages 15-16.

Exhibit C – Declaration under 37 C.F.R. §1.132 executed by Dr. Jens B. Nielsen with Exhibits 1-2 attached submitted on June 18, 2009 and February 1, 2010; entered into the record by the Examiner in the Office Action mailed May 10, 2010; pages 2 and 10.

Docket No.: 066662-0092 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Customer Number: 41552
Palsson, Bernhard	:	Confirmation Number: 1729
Application No.: 09/923,870	:	Group Art Unit: 1631
Filed: August 06, 2001	:	Examiner: Negin, Russell Scott

For: METHODS FOR IDENTIFYING DRUG TARGETS BASED ON GENOMIC SEQUENCE DATA

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jay D. Keasling, declare as follows:

1. I am a Professor in the Department of Chemical Engineering and Bioengineering at the University of California, Berkeley (UC Berkeley). I also hold the Hubbard Howe Jr. Distinguished Professor of Biochemical Engineering. I am the Acting Deputy Director of the Lawrence Berkeley National Laboratory and Synthetic Biology Engineering Research Center and am CEO of the Joint BioEnergy Institute. I joined the faculty of UC Berkeley in 1992 as an Assistant Professor. I became an Associate Professor in 1998 and was elevated to full professor in 2001. I served as Vice Chair of the Department of Chemical Engineering from 1999-2000 and have served as the Director and an Executive Committee Member of the UC BioSTAR Program since 2000.

2. Prior to joining the UC Berkeley faculty I obtained a Bachelors of Science majoring in chemistry and biology in 1986 from the University of Nebraska-Lincoln. I earned my Masters degree in 1988 and my Ph.D. in 1991, both in chemical engineering from the University of Michigan. From 1991-1992 I did a postdoctoral fellowship at Stanford University

EXHIBIT A

in Biochemistry. A copy of my curriculum vitae and a list of publications is attached as Exhibit 1.

3. I am an inventor or co-inventor on at least four U.S. patents and 16 U.S. applications. I am a founder of Amyris Biotechnologies and serve as the Chair of its Scientific Advisory Board. Amyris focuses on the microbial production of renewable fuels. I also am a founder of LS9 and Codon Devices. I have been a member of Genomatica's Scientific Advisory Board for the past year. My accomplishments in the fields of chemical engineering and synthetic biology have been reported in Time and Newsweek, and Discover magazine named me as the Scientist of the Year in 2006 for my work in synthetic biology, including treatments for malaria, AIDS, and cancer as well as discoveries of new fuel resources.

4. I am very familiar with stoichiometric models of metabolism and have read U.S. application serial no. 09/923,870, by Palsson. I also am very familiar with Dr. Palsson's work, including the publication that is the basis of this application (Edwards and Palsson, *Proc. Natl. Acad. Sci. U.S.A.*, 97:5528-33 (2000)). I understand that the invention described in this application is directed to constructing genome specific stoichiometric matrices that can be utilized with flux balance analysis for modeling metabolism. The application claims, in part, a method of simulating a metabolic capability by incorporating metabolic reactions through the use of genome information to assign function to metabolic proteins of unknown function.

5. I have read the Office Action mailed December 18, 2008. I understand that the claimed invention stands rejected for obviousness over the combination of references to Pramanik and Keasling., *Biotech. and Bioengineering* 56:398-421 (1997) in view of Blattner et al., *Science* 277:1453-69 (1997) and in view of Kunst et al., *Rev. in Microbiol.* 142:905-12 (1991). The Examiner appears to rely on Pramanik and Keasling for describing a stoichiometric model of *E. coli* metabolism and then combines it with Blattner et al. and Kunst et al., reporting the sequencing of *E. coli* and *B. subtilis* genomes, respectively, to conclude obviousness. The sequencing papers are used to support the Examiner's argument that one would have expected to be able to determine the function of genes encoding proteins of unknown function based on sequence comparisons with a different organism.

6. Pramanik and Keasling, the primary reference cited in the above rejection, is a publication from my laboratory and I am very familiar with this work. At the time of Dr. Palsson's invention, the *E. coli* genomic sequence had become available and my laboratory was actively working with stoichiometric models, including the model described in Pramanik and Keasling. We did not consider incorporating additional reaction information into the model based on the genomic sequence results for at least two reasons.

7. First, *in silico* models of metabolism such as that described in Pramanik and Keasling are complex computational models that are only as accurate as the information one includes in the model. There are a large number of metabolic enzymes encoded in the genome that are not used in metabolism. Incorporation of reactions based on genomic information would have included such unused enzymes and reactions in the model. One would have expected a large number of inaccurate fluxes to occur that would, in effect, travel everywhere throughout the network (i.e., wild or uncontrolled fluxes). As a result, the model would not have been predictive of an organism's metabolism and would have been expected to be much less accurate than the model described in Pramanik and Keasling. The fact that Dr. Palsson was able to construct a model incorporating reactions based primarily on genomic sequence information was surprising and unexpected because it did not result in wild fluxes nor decrease in accuracy compared to the Pramanik and Keasling model. Rather, the model yielded results that reflected actual cellular metabolism and was predictive despite the inclusion of more enzymes than what the network uses.

8. Second, incorporating reactions based on homology comparisons of unknown genes with metabolic genes in other organisms also was expected to yield inaccurate results. Although sequence identity comparisons can be predictive there are examples where identifications have been incorrect. Therefore, identification and assignment of some open reading frames as a putative metabolic enzyme was speculative and likely resulted in some incorrect assignments. These incorrect assignments can result in the inclusion of multiple reactions carrying out the same reaction, inclusion of unused reactions and the inclusion of non-metabolic enzymes into the metabolic network. For the reasons described above, incorporation of such inaccurate information was expected to generate wild fluxes if incorporated into a model such as that described in Pramanik and Keasling. It was surprising that one could. in fact,

I have read the Office Action mailed December 18, 2008.

incorporate putative metabolic enzymes and produce results that are predictive of cellular metabolism. Hence, the actual result of the claimed method is unexpected because this method is able to accurately predict metabolism even though reactions are incorporated based on deductions from sequence comparisons.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Jay D Keasling

12 June 2009

Jay D. Keasling

Date

SDO 140829-1.066662.0092

Jay D Keasling

Positions

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Postdoctorate, Biochemistry, 1991-1992, Stanford University
Ph.D., Chemical Engineering, 1991, University of Michigan
M.S., Chemical Engineering, 1988, University of Michigan
B.S., Chemistry and Biology, 1986, University of Nebraska, Lincoln

Professional Experience

Chief Executive Officer (2007 – present), Joint BioEnergy Institute, Emeryville, CA. *Acting Deputy Laboratory Director* (2009 – present), Lawrence Berkeley National Laboratory, Berkeley, CA. *Director* (2005 – 2009), Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA. *Senior Faculty Scientist* (2006 – present), *Faculty Scientist* (1992 – 2006), *Department Head* (2003 – present), Synthetic Biology Department, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA. *Professor* (2001 – present), *Vice Chair* (1999 – 2000), *Associate Professor* (1998 – 2001), *Assistant Professor* (1992 – 1998), Department of Chemical Engineering, University of California, Berkeley, CA. *Professor* (2004 – present), Department of Bioengineering, University of California, Berkeley, CA. *Director* (2006 – 2008), Synthetic Biology Engineering Research Center, University of California, Berkeley. *Director*, University of California Systemwide BioSTAR Project (2001 – 2003). *Executive Committee Chair*, University of California Discovery Grant Program (2003 – 2008). *Associate Editor, Biotechnology & Bioengineering* (2003 – 2005). *Postdoctoral Research Associate*, Department of Biochemistry, Stanford University School (1991 – 1992). *Research Assistant*, Department of Chemical Engineering, University of Michigan (1986 – 1991).

Honors

Inaugural *Biotech Humanitarian Award*, Biotechnology Industry Organization (BIO), 2009. *2009 University Lectures in Chemistry*, Department of Chemistry, Boston College, 2009. *The Sixteenth F. A. Bourke Distinguished Lecture in Biotechnology*, Center for Advanced Biotechnology and Department of Biomedical Engineering, Boston University, 2009. *Chancellor's Award for Public Service for Research in the Public Interest*, University of California, Berkeley, 2009. *2008 Britton Chance Distinguished Lecturer*, Department of Chemical and Biomolecular Engineering and Institute Medicine and Engineering, University of Pennsylvania, 2008. *2008 Patten Distinguished Seminar*, Department of Chemical Engineering, University of Colorado, 2008. *Sierra Section Recognition for Leadership in the Chemical Engineering Profession*,

EXHIBIT 1

American Institute of Chemical Engineers – Northern California Section, 2008. *Visionary Award,* Bay Bio, 2007. *Truman Lecturer*, Sandia National Laboratories, 2007. *Professional Progress Award,* American Institute for Chemical Engineers, 2007. Elected *Fellow of the American Academy for Microbiology*, 2007. *Research Project of the Year*, Northern California Section of the American Institute for Chemical Engineers, 2007. *Eastman Lectureship*, Department of Chemical Engineering, Georgia Tech University, 2007. *Scientist of the Year,* Discover Magazine, 2006. *Technology Pioneer,* World Economic Forum, 2005. *Seventh Annual Frontiers of Biotechnology Lecture,* Department of Chemical Engineering, Massachusetts Institute of Technology, 2005. *Blue-Green Lecturer,* Department of Chemical Engineering, University of Michigan & Department of Chemical Engineering and Materials Sciences, Michigan State University, 2005. *Inaugural Schwartz Lecturer*, Department of Chemical Engineering, Johns Hopkins University, 2003. *Allan P. Colburn Memorial Lecturer*, Department of Chemical Engineering, University of Delaware, 2002. Elected *Fellow of the American Institute of Medical and Biological Engineering*, 2000. *AIChE Award for Chemical Engineering Excellence in Academic Teaching,* Northern California Section of the American Institute for Chemical Engineers, 1999. *Chevron Young Faculty Fellowship*, Chevron, 1995. *CAREER Award*, National Science Foundation, 1995. *Zeneca Young Faculty Fellowship*, Zeneca Ltd., 1992-1997. *NIH Postdoctoral Fellowship*, Stanford University, 1991-1992. *Regents Scholarship,* The University of Nebraska, 1982-1986. *Graduation with High Distinction,* The University of Nebraska, 1986.

Memberships Phi Beta Kappa, American Chemical Society, American Institute of Chemical Engineers, American Society for Microbiology, American Institute of Medical and Biological Engineering

Refereed Journal Publications

1. J. D. Keasling and B. O. Palsson. 1989. "On the kinetics of plasmid replication." *J. Theor. Biol.* **136:**487-492.
2. J. D. Keasling and B. O. Palsson. 1989. "ColE1 plasmid replication: a simple kinetic description from a structured model." *J. Theor. Biol.* **141**:447-461.
3. B. O. Palsson, J. D Keasling, and S. G. Emerson. 1990. "The regulatory mechanisms of human immunodeficiency virus replication predict multiple expression rates." *Proc. Natl. Acad. Sci. USA.* **87:**772-776.
4. J. D. Keasling, B. O. Palsson, and S. Cooper. 1991. "Cell-cycle-specific F'*lac* plasmid replication: regulation by cell size control of initiation." *J. Bacteriol.* **173**:2673-2680.
5. J. D. Keasling, B. O. Palsson, and S. Cooper. 1992. "Replication of the R6K plasmid during the *Escherichia coli* cell cycle." *J. Bacteriol.* **174**:1060-1062.
6. J. D. Keasling, B. O. Palsson, and S. Cooper. 1992. "Replication of prophage P1 is cell-cycle specific." *J. Bacteriol.* **174**:4457-4462.
7. J. D. Keasling, B. O. Palsson, and S. Cooper. 1992. "Replication of mini-F plasmids during the bacterial division cycle." *Res. Microbiol.* **143**:541-548.
8. J. D. Keasling, L. Bertsch, and A. Kornberg. 1993. "Guanosine pentaphosphate phosphohydrolase of *Escherichia coli* is a long-chain polyphosphatase." *Proc. Natl. Acad. Sci. USA* **90**:7029-7033.
9. J. D. Keasling and S. Cooper. 1994. "Analysis of plasmid replication during the bacterial division cycle." *Methods in Molecular Genetics* **3**:380-388.
10. T. R. Hupp, J. D Keasling, S. Cooper, and J. M. Kaguni. 1994. "Synthesis of DnaK protein during the division cycle of *Escherichia coli*." *Res. Microbiol.* **145**:99-109.
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14. N. Shapiro and J. D. Keasling. 1996. "The *recA* gene and cadmium toxicity in *Escherichia coli* K-12." *Microbios* **86**:23-26.
15. H. Kuo and J. D. Keasling. 1996. "A Monte Carlo simulation of plasmid replication during the bacterial division cycle." *Biotechnol. Bioeng.* **52**:633-647.
16. S. Keyhani, J. L. Lopez, D. S. Clark, and J. D. Keasling. 1996. "Intracellular polyphosphate content and cadmium tolerance in *Anacystis nidulans* R2." *Microbios* **88**:105-114.
17. P. Wong, S. Gladney, and J. D. Keasling. 1996. "A mathematical model of the *lac* operon: inducer exclusion, catabolite repression, and diauxic growth on glucose and lactose." *Biotechnol. Prog.* **13**:132-143.
18. S. J. Van Dien, S. Keyhani, C. Yang, and J. D. Keasling. 1997. "Manipulation of independent synthesis and degradation of polyphosphate in *Escherichia coli* for investigation of phosphate secretion from the cell." *Appl. Environ. Microbiol.* **63**:1689-1695.
19. J. Elmen, W. Pan, S. Y. Leung, A. Magyarosy, and J. D. Keasling. 1997. "Kinetics of toluene degradation by a nitrate-reducing bacterium isolated from a groundwater aquifer." *Biotechnol. Bioeng.* **55**:82-90.
20. T. A. Carrier and J. D. Keasling. 1997. "Engineering mRNA stability in *E. coli* by the addition of synthetic hairpins using a 5' cassette system." *Biotechnol. Bioeng.* **55**:577-580.
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135. M. C. Y. Chang, R. A. Eachus, W. Trieu, D.-K. Ro, and J. D. Keasling. 2007. "Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s." *Nature Chem. Biol.* **3**:274-277.
136. Y. J. Tang, R. Chakraborty, H. G. Martin, J. Chu, T, C. Hazen, and J. D. Keasling. 2007. "Flux analysis of central metabolic pathways in *Geobacter metallireducens* during reduction of soluble Fe(III)-nitrilotriacetic acid." *Appl. Environ. Microbiol.* **73**:3859-3864.
137. S. H. Yoon, H. M. Park, J. E. Kim, S. H. Lee, M. S. Choi, J. Y. Kim, D. K. Oh, J. D. Keasling, and S. W. Kim. 2007. "Increased beta-carotene production in recombinant *Escherichia coli* harboring an engineered isoprenoid precursor Pathway with mevalonate addition." *Biotechnol. Prog.* 23:599-605.
138. A. Mukhopadhyay, A. M. Redding, M. P. Joachimiak, A. P. Arkin, S. E. Borglin, P. S. Dehal, R. Chakraborty, J. T. Geller, T. C. Hazen, Q. He, D. C. Joyner, V. J. J. Martin, J. D. Wall, Z. K. Yang, J. Zhou, and J. D. Keasling. 2007. "Cell wide responses to low oxygen exposure in *Desulfovibrio vulgaris* Hildenborough." *J. Bacteriol.* **189**:5996-6010.
139. S. K. Lee, H. H. Chou, B.F. Pfleger, J. D. Newman, Y. Yoshikuni, and J. D. Keasling. 2007. "Directed evolution of AraC for improved compatibility of arabinose and lactose-inducible promoters." *Appl. Environ. Microbiol.* **73**:5711-5715.
140. S. T. Withers, S. S. Gottlieb, B. Lieu, J. D. Newman, and J. D. Keasling. 2007. "Identification of isopentenol biosynthetic genes from *Bacillus subtilis* using isoprenoid precursor toxicity." *Appl. Environ. Microbiol.* **73**:6277-6283.
141. K. D. McMahon, S. Yilmaz, S. He, D. L. Gall, D. Jenkins, and J. D. Keasling. 2007. "Polyphosphate kinase genes from full-scale activated sludge plants." *Appl. Microbiol. Biotechnol.* **77**:167-173.
142. B. M. T. da Costa, K. Cornish, and J. D. Keasling. 2007. "Manipulation of intracellular magnesium levels in *Saccharomyces cerevisiae* with deletion of magnesium transporters." *Appl. Microbiol. Biotechnol.* **77**:411-425.
143. R. E. Osterhout, I. A. Figueroa, J. D. Keasling, and A. P. Arkin. 2007. "Global analysis of host response to induction of a latent bacteriophage." *BMC Microbiology*. **7**:82.
144. S. Mahendra, C. J. Petzold, E. E. Baidoo, J. D. Keasling, and L. Alvarez-Cohen. 2007. "Identification of the intermediates of *in vivo* oxidation of 1,4-dioxane by monooxygenase-containing bacteria." *Environ. Sci. Technol.* **144**:7330-7336.
145. P. F. Suthers, A. P. Burgard, M. S. Dasika, F. Nowroozi, S. Van Dien, J. D. Keasling, and C. D. Maranas. 2007. "Metabolic flux elucidation for large-scale models using ^{13}C labeled isotopes." *Metab. Eng.* **9**:387-405.
146. V. Hale, J. D. Keasling, N. Renninger, and T. T. Diagana. 2007. "Microbially derived artemisinin: a biotechnology solution to the global problem of access to affordable anti-malarial drugs." *Am. J. Trop. Med. Hyg.* **77**:198-202.
147. H. W. Blanch, P. D. Adams, K. M. Andrews-Cramer, W. B. Frommer, B. A. Simmons, and J. D. Keasling. 2008. "Addressing the need for alternative transportation fuels: the Joint BioEnergy Institute." *ACS Chem. Biol.* **3**:17-20.

148. W. D. Marner, A. S. Shaikh, S. J. Muller, and J. D. Keasling. 2008. "Morphology of artificial silica matrices formed via autosilification of a silaffin/protein polymer chimera." *Biomacromol.* **9**:1-5.
149. J. D. Keasling. 2008. "Synthetic biology for synthetic chemistry." *ACS Chem. Biol.* **3**:64-76.
150. A. S. Shaikh, Y, J. Tang, A. Mukhopadhyay, and J. D. Keasling. 2008. "Isotopomer distributions in amino acids from a high expressed protein as a proxy for those from total protein." *Anal. Chem.* **80**:886-890.
151. J. D. Keasling and H. Chou. 2008, "Metabolic engineering delivers next-generation biofuels." *Nat. Biotechnol.* **26**:298-299.
152. D. M. Wolf, L. Fontaine-Bodin, I. Bischofs, G. Price, J. Keasling, and A. P. Arkin. 2008. "Memory in microbes: quantifying history-dependent behavior in a bacterium." *PLoS ONE.* **3**:e1700.
153. J. Kirby, D. W. Romanini, E. M. Paradise, and J. D. Keasling. 2008. "Engineering triterpene production in *Saccharomyces cerevisiae* – beta-amyrin synthase from *Artemisia annua*." *FEBS J.* **275**:1852-1859.
154. L. Kizer, D. J. Pitera, B. Pfleger, and J. D. Keasling. 2008. "Functional genomics for pathway optimization: application to isoprenoid production." *Appl. Environ. Microbiol.* **74**:3229-3241.
155. E. E. K. Baidoo, P. I. Benke, C. Neususs, M. Pelzing, G. Kruppa, J. A. Leary, and J. D. Keasling. 2008. "Capillary electrophoresis-Fourier transform ion cyclotron resonance mass spectrometry for the identification of cationic metabolites via a pH-mediated stack-transient isotrachophoretic method." *Anal. Chem.* **80**:3112-3122.
156. E. M. Paradise, J. Kirby, R. Chan, and J. D. Keasling. 2008. "Redirection of flux through the FPP branch-point in *Saccharomyces cerevisiae* by downregulating squalene synthase." *Biotechnol. Bioeng.* **100**:371-378.
157. A. Mukhopadhyay, A. M. Redding, B. J. Rutherford, and J. D. Keasling. 2008. "Importance of systems biology in engineering microbes for biofuel production." *Curr. Opin. Biotechnol.* **19**:228-234.
158. D. E. Garcia, E. E. Baidoo, P. I. Benke, F. Pingitore, Y. J. Tang, S. Villa, and J. D. Keasling. 2008. "Separation and mass spectrometry in microbial metabolomics." *Curr. Opin. Microbiol.* **11**:233-239.
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160. S. P. Gaucher, A. M. Redding, A. Mukhopadhyay, J. D. Keasling, and A. K. Singh. 2008. "Post-translational modifications of *Desulfovibrio vulgaris* Hildenborough sulfate reduction pathway proteins." *J. Proteome Res.* 7:2320-2331.
161. Y. Yoshikuni, J. A. Dietrich, F. F. Nowroozi, P. C. Babbitt, and J. D. Keasling. 2008. "Redesigning enzymes based on adaptive evolution for optimal function in synthetic metabolic pathways." *Chem. Biol.* **15**:607-618.
162. T. S. Ham, S. K. Lee, J. D. Keasling, and A. P. Arkin. 2008. "Design and construction of a double inversion recombination switch for heritable sequential genetic memory." *Plos One* **3**:e2815.
163. J. Kirby and J. D. Keasling. 2008. "Metabolic engineering of microorganisms for isoprenoid production." *Nat. Prod. Rep.* **25**:656-661.
164. J. D. Keasling. 2008. "From yeast to alkaloids" *Nat. Chem. Biol.* **4**:524-525.
165. S. K. Lee and J. D. Keasling. 2008. "Heterologous protein production in *Escherichia coli* using the propionate-inducible pPro system by conventional and auto-induction methods." *Prot. Exp. Purif.* **61**:197-203.
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167. W. Shui, L. Sheu, J. Liu, B. Smart, C. J. Petzold, T. Y. Hsieh, A. Pitcher, J. D. Keasling, and C. R. Bertozzi. 2008. "Membrane proteomics of phagosomes suggests a connection to autophagy." *Proc. Natl. Acad. Sci. USA* **105**:16952-16957.

168. S. K. Lee, H. Chou, T. S. Ham, T. S. Lee and J. D. Keasling. 2008. "Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels." *Curr. Opin. Biotechnol.* **19**:556-563.
169. D.-K. Ro, M. Ouellet, E. M. Paradise, H. Burd, D. Eng, C. J. Paddon, J. D. Newman, and J. D. Keasling. 2008. "Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of antimalarial drug precursor, artemisinic acid." *BMC Biotechnol.* **8**:83 (doi:10.1186/1472-6750-8-83).
170. E. J. Steen, R. Chan, N. Prasad, S. Myers, C. J. Petzold, A. Redding, M. Ouellet, and J. D. Keasling. 2008. "Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol." *Microb. Cell Fact.* **7**:36.
171. J. R. Anthony, L. C. Anthony, F. Nowroozi, G. Kwon, J. D. Newman, and J. D. Keasling. 2009. "Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *E. coli* for production of the anti-malarial drug precursor amorpha-4,11-diene." *Met. Eng.* **11**:13-19.
172. D. Lubertozzi and J. D. Keasling. 2009. "Developing Aspergillus as a host for heterologous expression." *Biotechnol. Adv.* **27**:53-75.
173. W. Shui, S. A. Gilmore, L. Sheu, J. Liu, J. D. Keasling, and C. R. Bertozzi. 2009. Quantitative proteomic profiling of host-pathogen interactions: the macrophage response to *Mycobacterium tuberculosis*. *J. Proteome Res.* **8**:282-289.
174. Y. J. Tang, H. G. Martin, P. S. Dehal, A. Deutschbauer, X. Llora, A. Meadows, A. Arkin, and J. D. Keasling. 2009. "Metabolic flux analysis of *Shewanella* spp. reveals evolutionary robustness in central carbon metabolism." *Biotechnol. Bioeng.* **102**:1161-1169.
175. Y. J. Tang, H. Garcia Martin, S. Myers, S. Rodriguez, E. E. K. Baidoo, and J. D. Keasling. 2009. "Advances in analysis of microbial metabolic fluxes via ^{13}C isotopic labeling." *Mass Spec. Rev.* **28**:362-375.
176. H. Tsuruta, C. J. Paddon, D. Eng, J. R. Lenihan, T. Horning, L. C. Anthony, R. Regentin, J. D. Keasling, N. S. Renninger, and J. D. Newman. 2009. "High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*." *PLOS One* **4**:e4489 doi:10.1371.
177. Y. J. Tang, R. Sapra, D. Joyner, T. C. Hazen, S. Myers, D. Reichmuth, H. Blanch, and J. D. Keasling. 2009. "Analysis of metabolic pathways and fluxes in a newly discovered thermophilic and ethanol-tolerant *Geobacillus* strain." *Biotechnol. Bioeng.* **102**:1377-1386.
178. W. D. Marner, A. Shaikh, S. J. Muller, and J. D. Keasling. 2009. "Enzyme immobilization via silaffin-mediated autoencapsulation in a biosilica support." *Biotech. Prog.* **25**:417-423.
179. J. Dietrich, Y. Yoshikuni, K. Fisher, F. Woolard, D. Ockey, D. McPhee, N. Renninger, M. Chang, D. Baker, and J. D. Keasling. 2009. "A novel semi-biosynthetic route for artemisinin production using engineered substrate-promiscuous P450BM3." *ACS Chem. Biol.* **4**:261-267.
180. J. Kirby and J. D. Keasling. 2009. "Biosynthesis of plant isoprenoids: perspectives for microbial engineering." *Annu. Rev. Plant Biol.* **60**:335.355.
181. D. A. Elias, A. Mukhopadhyay, M. P. Joachimiak, E. C. Drury, A. M. Redding, H. C. Yen, M. W. Fields, T. C. Hazen, A. P. Arkin, J. D. Keasling, J. D. Wall. 2009. "Expression profiling of hypothetical genes in *Desulfovibrio vulgaris* leads to improved functional annotation." *Nucl. Acids Res.* In press.
182. Y. J. Tang, W. Shui, S. Myers, X. Feng, C. Bertozzi, J. D. Keasling. 2009. "Central metabolism in Mycobacterium smegmatis during the transition from O(2)-rich to O (2)-poor conditions as studied by isotopomer-assisted metabolite analysis." *Biotechnol. Prog.* In press.

Refereed Conference Proceedings

1. S. T. Sharfstein and J. D. Keasling. 1994. "Polyphosphate metabolism in *Escherichia coli*." *Ann. N. Y. Acad. Sci.* **745**:77-92.
2. J. D. Keasling. 1997. "Regulation of intracellular toxic metals and other cations by hydrolysis of polyphosphate." *Ann. N. Y. Acad. Sci.* **829**:242-249.

3. D. G. Bolesch, R. B. Nielsen, and J. D. Keasling. 1997. "Complete reductive dechlorination of trichloroethene by a groundwater microbial consortium." *Ann. N. Y. Acad. Sci.* **829**:97-102.
4. D. Szykowny and J. D. Keasling. 1997. "Kinetics of BTEX degradation by a nitrate-reducing mixed culture." *Ann. N. Y. Acad. Sci.* **829**:135-141.
5. R. B. Nielsen and J. D. Keasling. 1998. "Anaerobic degradation of PCE and TCE DNAPLs by groundwater microorganisms." In *Bioremediation and Phytoremediation: Chlorinated and Recalcitrant Compounds*, G. B. Wickramanayake and R. E. Hinchee, eds. Battelle Press, Columbus, OH. pp. 97-102.
6. J. D. Keasling, J. Benemann, J. Pramanik, T. A. Carrier, K. L. Jones, and S. J. Van Dien. 1998. "A toolkit for metabolic engineering of bacteria: application to hydrogen production." In *Biohydrogen*, O. Zaborsky, ed. pages 87-97. Plenum Publishing, New York, NY.
7. S. W. Bang, D. S. Clark, and J. D. Keasling. 1999. "Heavy metal decontamination by engineered genetic cassettes." Proceedings of the World Congress of Korean Scientists and Engineers. Pgs. 449-455. The Korean Federation of Science and Technology Societies. Seoul, Korea.
8. M. M. Maharbiz, R. T. Howe, J. D. Keasling. 2000. "Silicon microbial bioreactor arrays." Proceedings of the IEEE-EMBS Special Topic Conference on MicroTechnologies in Medicine and Biology, Paris, France, October 12-14, 2000. pp. 165-170.
9. M.M. Maharbiz, W.J. Holtz, S. Sharifzadeh, J.D. Keasling and R.T. Howe. 2002. "A microfabricated electrochemical oxygen generator for high-density cell culture arrays." Solid-State Sensor, Actuator and Microsystems Workshop, Hilton Head Island, SC, June 2-6 2002. pp. 259-264.
10. J.D. Keasling, 2005. "The promise of synthetic biology." *The Bridge* **35**(4):18-21. Presented at the National Academy of Engineering, U.S. Frontiers of Engineering meeting, GE Global Research Center, Niskayuna, NY, September 22-24.
11. C. A. Voigt and J. D. Keasling. 2005. "Programming cellular function." *Nat. Chem. Biol.* **1**:304-307. Life Engineering Symposium, San Francisco, CA. August 19-20, 2005.

Refereed Book Chapters

1. J. D. Keasling, T. A. Carrier, K. L. Jones, J. Pramanik, and S. J. Van Dien. 1999. "New tools for metabolic engineering of *Escherichia coli*." In *Metabolic Engineering*, S.-Y. Lee and E. T. Papoutsakis, eds. Marcel Dekker, New York, NY.
2. C. D. Smolke, V. J.J. Martin, and J. D. Keasling. 2004. "Tools for Metabolic Engineering in *Escherichia coli*." In *Protein Expression Technologies: Current Status and Future Trends*. In *Protein expression technologies. Current Status and Future Trends*, F. Baneyx, ed. Horizon Bioscience, Norfolk, UK. Pages 149-197.
3. G. Y. Wang, R. D. Laidlaw, J. H. Marshall, and J. D. Keasling. 2004. "Metabolic engineering of fungal secondary metabolite pathways." In *Handbook of Industrial Mycology*, Z. An, ed. Marcel Dekker, New York, NY. Pages 635-666.
4. M. de la Pena Mattozzi, Y. Kang, J. D. Keasling. 2009. "Feast: Choking on Acetyl-CoA, the Glyoxylate Shunt, and Acetyl-CoA-Driven Metabolism." In *Handbook of Hydrocarbon and Lipid Microbiology*. Springer DE
5. J. D. Keasling. 2009. "Microbial production of isoprenoids." In *Handbook of Hydrocarbon and Lipid Microbiology*. Springer DE

Patents

1. J. D. Keasling, D. G. Bolesch, and T. Delfino. 1995. "Reductive dehalogenation of organic halides in contaminated groundwater." US Patent No. 6,150,157.
2. J. D. Keasling, V. Martin, D. Pitera, S.-W. Kim, S. T. Withers III, Y. Yoshikuni, J. Newman, A. V. Khlebnikov. 2007. "Biosynthesis of isopentenyl pyrophosphate." US Patent No. 7,172,886.
3. J. D. Keasling, J. D. Newman, D. J. Pitera. 2007. "Method for enhancing production of isoprenoid compounds." US Patent No. 7,183,089.
4. J. D. Keasling, V. Martin, D. Pitera, S. T. Withers III, J. Newman. 2007. "Biosynthesis of amorpha-4,11-diene." US Patent No. 7,192,751.

Pending patent applications

5. K. K. Reiling, J. D. Newman, S. T. Withers, D. J. Pitera, J. D. Keasling, V. J. J. Martin. 2004. "Methods for identifying biosynthetic pathway gene product."
6. J.D. Keasling, J. Kirby, E. Paradise. 2004 "Metabolically Engineered Yeast for Terpene Production."
7. D.K. Ro, K. Newman, M. Chang, Y. Shiba, E. Paradise, J.D. Keasling, M. Ouellet, R. Eachus, K. Ho. 2005. "Biosynthesis of Artemisinic Acid, a Key Intermediate in the Artemisinin Pathway."
8. J.D. Keasling, S. Lee. 2005 "Inducible Expression Vectors and Methods of Use Thereof."
9. J.D. Keasling, J.D. Newman, D.J. Pitera. 2005. "Method for Enhancing Production of Isoprenoid Compounds."
10. Y. Shiba, J. Kirby, K.L. Newman, S.T. Withers, III, E. Paradise, J.D. Keasling. 2005. "Improvement of isoprenoid production level in yeast."
11. D.K. Ro, K. Newman, E. Paradise, J.D. Keasling, M. Ouellet, R. Eachus. 2005. "Polynucleotides Encoding Isoprenoid Modifying Enzymes and Methods of Use Thereof."
12. Y. Shiba, J. Kirby, E. M. Paradise, J.D. Keasling. 2005."Genetically Modified Host Cells and Use of Same for Producing Isoprenoid Compounds."
13. J. Kirby, J.D. Keasling, A. Shaikh, M. Mattozzi, E. Paradise. 2005. "Engineering of the DXP Pathway in Yeast for Isoprenoid Production."
14. M. Chang, R. Eachus, D.K. Ro, Y. Yoshikuni, J.D. Keasling. 2005. "Nucleic Acids Encoding Isoprenoid Precursor Modifying Enzymes and Methods of Use Thereof."
15. J. Keasling, C. Paddon, B. Pfleger, D. Pitera. 2006. "Nucleic Acids Encoding Modified Cytochrome P450 Enzymes and Methods of Use Thereof."
16. J. D. Keasling, F. Nowroozi, D. Pitera, J. D. Newman, J. Anthony, L. Anthony. 2006. "Determination of limiting genes in the Amorphadiene Pathway and development of Modular Vectors to facilitate this goal."
17. W.D. Marner II, A.S. Shaikh, J.D. Keasling, W.J. Holtz, S.T. Withers, J.L. Kizer. 2006. "A method for functionalizing proteins to incorporate auto-biosilification activity."
18. J.D. Keasling, J. Kirby. 2005. "Metabolically Engineered Yeast for Terpene Production."
19. D. Pitera, J.D. Newman, J.L. Kizer, J.D. Keasling, B. Pfleger. 2006. "Methods and Compositions for Modulating Fatty Acid Biosynthesis and Uses Thereof."
20. Y. Yoshikuni, J.D. Keasling. 2005. "Methods of generating protein variants with altered function."
21. J. A. Dietrich, Y. Yoshikuni, J.D. Keasling, M.C. Chang. 2006. "Production of artemisinic-11,12-epoxide by engineered cytochrome $P450_{BM3}$."

Invited Presentations

1. University of California at Davis, Department of Chemical Engineering, Davis, CA, May 1993
2. Genentech, South San Francisco, CA, January 1994.
3. Zeneca Bio-products, Billingham, England, November 1994.
4. University of California at Santa Barbara, Department of Chemical Engineering, Santa Barbara, CA, October, 1994.
5. Chiron, Emeryville, CA, May 1996.
6. Stanford University, Department of Chemical Engineering, Stanford, CA May 1997.
7. Society for Industrial Microbiology, National Meeting, Reno, NV, August 1997.
8. 7th Biochemical Engineering conference, Seoul, Korea, September 1997.
9. California Water Environment Association. 1998 Annual CWAE Conference. April 1998.
10. Merck and Co., July 1998.
11. Institute for Biological Engineering, Annual Meeting, Orlando, FL, July 1998.
12. California Institute of Technology, Department of Environmental Engineering Science, Pasadena, CA. September 30, 1998.
13. University of Wisconsin, Department of Chemical Engineering, Madison, WI. October 1998.

14. University of Michigan, Department of Chemical Engineering, Ann Arbor, MI. October 1998.
15. University of Michigan, Department of Microbiology and Immunology, Ann Arbor, MI. October 1998.
16. University of Toledo, Department of Bioengineering, Toledo, OH. January 1999.
17. Chiron, Emeryville, CA. June 1999.
18. Merck and Co., Bioprocess Research and Development. June 1999.
19. Enzyme Engineering XV, Kona, Hawaii. October 1999.
20. Massachusetts Institute of Technology, Department of Chemical Engineering. November 1999.
21. University of California at Irvine, Department of Chemical and Environmental Engineering. February 2000.
22. International symposium on Modern problems of Microbial Biochemistry and Biotechnology. Pushchino, Russia. June 2000.
23. International Society for Environmental Biotechnology meeting. Kyoto, Japan. July 2000.
24. Biotechnology 2000. Berlin, Germany. August 2000.
25. Princeton University, Department of Chemical Engineering. September 2000.
26. Metabolic Engineering and Directed Evolution. British Biotechnology Research Council. London, England. November 2000.
27. University of Minnesota, Department of Chemical Engineering. December 2000.
28. Pacifichem. Honolulu, Hawaii. December 2000.
29. World Congress on Enzyme Technologies. San Diego, CA. February 26, 2001.
30. American Society for Microbiology Annual Meeting. Orlando, FL. May 19, 2001.
31. Genomatica. San Diego, CA. October 26, 2001.
32. University College London, Department of Bioprocess Engineering. London, UK. October 22, 2001.
33. University of Wisconsin, Department of Chemical Engineering. Madison, WI. October 30, 2001.
34. Actinides 2001. Hayama, Japan. November 5, 2001.
35. Princeton University, Department of Chemical Engineering. Princeton, NJ. December 7, 2001.
36. Stanford University, Department of Civil and Environmental Engineering. Stanford, CA. February 8, 2002.
37. University of Maryland, Department of Chemical Engineering. College Park, Maryland. February 25, 2002.
38. Microbia. Cambridge, MA. February 27, 2002.
39. Diversa. San Diego, CA. March 13, 2002.
40. Kosan Biosciences. Hayward, CA. March 29, 2002.
41. University of Washington, Department of Chemical Engineering. Seattle, WA. April 22, 2002.
42. National Research Center, Biotechnology Research Institute. Montreal, Canada. June 3, 2002.
43. Sandia National Laboratory. August 27, 2002.
44. Tenth International Small Genomes Conference. Lake Arrowhead, CA. September 9, 2002.
45. City College of New York, Department of Chemical Engineering. New York, NY. September 30, 2002
46. Polytechnic University, Department of Chemical Engineering. Brooklyn, NY. October 2, 2002.
47. University of Delaware, Department of Chemical Engineering (Allan P. Colburn Memorial Lecture). Newark, DE. November 2, 2002.
48. American Institute of Chemical Engineers National Meeting. Indianapolis, IN. November 5, 2002.
49. Firmenich. Geneva, Switzerland. November 7, 2002.
50. University of Michigan, Cellular Biotechnology Program. January 13, 2003.

51. Metabolic Engineering Working Group, National Science Foundation, Arlington, VA. January 31, 2003.
52. Synthetic Biology. SRI International. Menlo Park, CA. March 3-4, 2003.
53. University of California, San Diego, Department of Chemistry, La Jolla, CA. April 4, 2003.
54. Annual Meeting of the Society for Biochemistry and Molecular Biology (ASBMB), San Diego, CA. April 15, 2003.
55. Terpnet Meeting, University of Kentucky, Lexington, KY. May 15, 2003.
56. Johns Hopkins University, Department of Chemistry, Baltimore, MD. May 19, 2003.
57. Society of Industrial Microbiology Annual Meeting, Minneapolis, MN. August 10, 2003.
58. University of California, Berkeley, Department of Chemistry, Berkeley, CA. September 2, 2003.
59. University of Nebraska, Lincoln, Department of Chemistry, Lincoln, NE. September 12, 2003.
60. University of Illinois, Department of Chemical Engineering, Urbana-Champaign, IL. September 29, 2003.
61. Rice University, Department of Chemical Engineering, Houston, TX. October 9, 2003.
62. University of Colorado, Department of Chemical Engineering, Boulder, CO. October 14, 2003.
63. AIChE National Meeting, San Francisco, CA. November 17, 2003.
64. Thirteenth ISBA Meeting, Melbourne, Australia. December 1-5, 2003.
65. Eidgenossische Technische Hochschule, Department of Chemistry, Zurich, Switzerland. March 22, 2004.
66. PSI Protein Production and Crystallization Workshop, National Institute of General Medical Sciences, Natcher Conference Center, Bethesda, Maryland. March 29, 2004.
67. Illinois Institute of Technology, Department of Chemical Engineering, Chicago, IL. April 28, 2004.
68. Biotech Summit, Berkeley, CA. May 10, 2004.
69. Biological Input-Output Systems, DARPA, Boston MA. June 14, 2004.
70. Biotec 2004, Oviedo, Spain. July, 19, 2004.
71. Society for Industrial Microbiology, Anaheim, CA. July 25, 2004.
72. American Chemical Society, Philadelphia, PA. August 22, 2004.
73. Cornell University, Department of Chemical Engineering, Ithaca, NY. September 13, 2004.
74. Purdue University, Department of Chemical Engineering, West Lafayette, IN. September 14, 2004.
75. Metabolic Engineering V, Lake Tahoe, CA. September 19, 2004.
76. Small Genomes Meeting, Lake Arrowhead, CA. September 26, 2004.
77. Council for the Advancement of Science Writing, Fayetteville, AK. November 8, 2004.
78. BioAgenda, Palm Springs, CA. December 7, 2004.
79. The Crossroads of Biotechnology 2005, Montreal, Canada, February 8, 2005.
80. USDA-ARS Commercial Strategic Rubber from Crop Plants and Bioreactors Third Annual Meeting, Albany, CA. February 17-18, 2005.
81. SynBio 2005 International Conference, Seoul, Korea, February 23, 2005.
82. 229th ACS National Meeting, San Diego, CA. March 12, 2005.
83. American Society for Microbiology 105th General Meeting, Atlanta, GA. June 9, 2005.
84. Gordon Research Conference "Plant Metabolic Engineering", Tilton, NH. July 13-15, 2005.
85. International Union of Microbiological Societies (IUMS), San Francisco, CA. July 27, 2005.
86. Manipulation of Biological Systems Conference, McLean, VA. July 28, 2005.
87. 2005 SIMS Annual Meeting, Chicago, IL. August 22-23, 2005.
88. 13th Annual International Conference on Microbial Genomes, Madison, WI. September 13-15, 2005.
89. National Academy of Engineering 11th Annual US Frontiers of Engineering Symposium, Niskayuna, NY. September 22-24. 2005.

90. University of California, Santa Barbara, Department of Chemical Engineering, Santa Barbara, CA. October 6, 2005.
91. ICSB 2005, Boston, MA. October 20-22, 2005.
92. IBM Academy of Technology Annual Meeting, Burlingame, CA. November 2, 2005.
93. University of Michigan, Department of Chemical Engineering, East Lansing, MI. November 8-12, 2005.
94. Pacifichem 2005, Honolulu, Hawaii, December 16-18, 2005.
95. 2006 Institute of Biological Engineering Conference, Tucson, AZ. March 9-12, 2006.
96. University of Virginia, 2006 Symposium, Charlottesville, VA. April 10-11, 2006.
97. University of San Diego, San Diego, CA April 13-14, 2006.
98. Stanford University, Stanford, CA. May 9, 2006.
99. DuPont Central Research and Development, Wilmington, DE. June 1-3, 2006.
100. CNN Future Summit "Of Man and Machine", Singapore, June 11-15, 2006.
101. IUCRP Fellows Seminar, UC San Diego, San Diego, CA, July 11, 2006.
102. SIMS 2006 Annual Meeting, Baltimore, MD, July 30-31, 2006.
103. California Commonwealth Club's INFORUM, San Francisco, CA. August 7, 2006.
104. Seminar, University of Minnesota, Dept. of Chemical Engineering and Material Sciences, Minneapolis, MN Sept. 11-12, 2006.
105. Seminar, University of California, Irvine, Synthetic Biology Department, Irvine, CA, Sept. 14-15, 2006.
106. 14th Annual International Meeting on Microbial Genomics, Lake Arrowhead, CA, September 24-28, 2006.
107. IBOS Conference, Nunspeet, The Netherlands, September 27 – 30, 2006.
108. Metabolic Engineering VI: From recDNA towards Engineering Biological Systems, Noordwijkerhout, The Netherlands, October 1-5, 2006.
109. UC Berkeley Homecoming Seminar, Berkeley, CA. October 6, 2006.
110. Contra Costa College, San Pablo, CA, October 13, 2006.
111. Invited Presentation, 3rd International *E. coli* Alliance Conference, Jeju, South Korea, November 1-3, 2006.
112. Seminar, IBM Almaden Research Center, San Jose, CA, November 7, 2006.
113. Invited Presentation, William L. Brown Symposium, Missouri Botanical Garden, St. Louis, MO. November 10-11, 2006.
114. Seminar, University of California, San Francisco, Department of Biophysics and Chemistry, San Francisco, CA, November 16, 2006.
115. Invited Presentation, Keystone Symposium, Drugs Against Protozoan Parasites, Lake Tahoe, CA, January 28, 2007.
116. Keynote Address, Biotechnology and Biological Sciences Research Council, BBSRC Workshop in Synthetic Biology, Alexandra House, Wroughton, Swindon, UK, February 8, 2007.
117. Seminar, Stanford University, Department of Microbiology, Stanford, CA, February 16, 2007.
118. Keynote Address, The World Congress on Industrial Biotechnology & Bioprocessing, Biotechnology Industry Organization, Orlando, FL, March 23, 2007.
119. Keynote Address, Joint Genome Institute User's Meeting, Walnut Creek, CA, March 28, 2007.
120. Seminar, University of Missouri, Columbia, Department of Biochemistry, Columbia, MO, April 13, 2007.
121. Panelist, Burrill General Partners Meeting, San Francisco, CA, April 17, 2007.
122. Keynote Address, Recomb 2007, Oakland, CA, April 23, 2007.
123. Seminar, Harvard Malaria Symposium, Harvard University, Cambridge, MA, April 24, 2007.
124. Seminar, Georgia Tech University, Center for the Study of Systems Biology, Atlanta, GA, May 2, 2007.
125. Seminar, Georgia Tech University, Department of Chemical Engineering, Atlanta, GA, May 3, 2007.
126. Seminar, Northern California AIChE, Berkeley, CA, May 15, 2007.

127. Seminar, University of British Columbia, Michael Smith Laboratories, Vancouver, British Columbia, Canada, May 17, 2007.
128. Seminar, Congressional Biomedical Research Caucus, Washington, D.C., May 23, 2007.
129. Seminar, PARC Forum, Palo Alto Research Center, Palo Alto, CA, May 24, 2007.
130. Seminar, Harvard University, Department of Chemistry, Cambridge, MA, May 31, 2007.
131. Seminar, Kavli Futures Symposium, Ilulissat, Greenland, June 13, 2007.
132. Seminar, University of Manchester, Manchester Institute of Biotechnology, Manchester, UK, July 12, 2007.
133. Presentation, Biochemical Engineering XV, Quebec City, Canada, July 12, 2007.
134. Presentation, Natural Products Gordon Research Conference, Tilton, NH, July 25, 2007.
135. Presentation, Society for Industrial Microbiology Meeting, Denver, CO, July 29, 2007.
136. Presentation, Energy Modeling Forum, Workshop on Climate Impacts and Integrated Assessment, Snowmass, CO, August 1, 2007.
137. Keynote Address, 10th Functional Genomics Meeting on Synthetic Biology, Goteborg, Sweden, August 28, 2007.
138. Presentation, KI International Symposium Future Design, Korean Advanced Institute for Science and Technology, Daejeon, Korea, September 6, 2007.
139. Keynote Address, Enzyme Engineering XIX, Harrison Hot Springs, British Columbia, Canada, September 23, 2007.
140. Presentation, Metabolic Engineering Meeting, Mathematical Biosciences Institute, Ohio State University, Columbus, OH, September 24, 2007.
141. Keynote Address, Frontiers in Transgenesis, Danforth Center, St. Louis, OH, September 28, 2007.
142. Seminar, Rice University, Department of Bioengineering, Houston, TX, October 10, 2007.
143. Presentation, Malaria Forum, Bill & Melinda Gates Foundation, Seattle, WA, October 17, 2007.
144. Presentation, Pop!Tech, Camden, ME, October 20, 2007.
145. Presentation, Energy Roundtable, Stanford University, Hoover Institute, Stanford, CA, November 20, 2007.
146. Presentation, Biological and Environmental Research Advisory Committee (BERAC), Washington, DC, November 29, 2007.
147. Harry S. Truman Award Lecture, Sandia National Laboratories, Albuquerque, NM, December 5, 2007.
148. Presentation, International Conference on Cellular & Molcular Bioengineering, Nanyang Technological University, Singapore, December 10, 2007.
149. Presentation, Symposium on Future Directions in Research at the Intersection of the Physical and Life Sciences (RIPLS), National Academy of Science, Washington, D.C., December 19, 2007.
150. Keynote Address, Technology Innovation Conference, Novozymes, Copenhagen, Denmark, January 13, 2008.
151. Presentation, US-EC Energy Symposium Exact Name, San Francisco, CA, February 22, 2008.
152. Keynote Address, 6th TLL Life Sciences Symposium, Temasec Life Sciences Laboratories, Singapore National University, Singapore, January 25, 2007.
153. Presentation, Orinda Intermediate School, Orinda, CA, January 30, 2007.
154. Keynote Address, 12th Netherlands Biotechnology Conference, Ede, The Netherlands, March 14, 2007.
155. Presentation, Symposium on Synthetic Biology, University of Arizona, Tucson, AZ, March 19, 2008.
156. Seminar, Duke University, Department of Biochemistry, Durham, NC, March 21, 2008.
157. Seminar, Reliance Life Sciences, Mumbai, India, March 28, 2008.
158. Seminar, Council of Scientific and Industrial Research, New Dehli, India, March 30, 2008.
159. Seminar, University of Nevada, Reno, Department of Chemical Engineering, Reno, NV, April 7, 2008.

160. Seminar, University of California, Berkeley, Department of Mechanical Engineering, Berkeley, CA, March 10, 2008.
161. Presentation, Targeting and Tinkering with Interaction Networks, Barcelona, Spain, April 15, 2008.
162. Presentation, Institute for Systems Biology, Seattle, WA, April 21, 2008.
163. Seminar, University of Washington, Department of Bioengineering, Seattle, WA, April 22, 2008.
164. Seminar, Sangamo Biosciences, Richmond, CA, April 25, 2008.
165. Presentation, Fifth Annual World Congress on Industrial Biotechnology & Bioprocessing, Chicago, IL, April 28, 2008.
166. Seminar, California Institute of Technology, Department of Bioengineering, Pasadena, CA, May 5, 2008.
167. Presentation, Khosla Ventures CEO Summit, location, May 7, 2008.
168. Seminar, Scripps Research Institute, Department of Chemistry, La Jolla, CA, May 8, 2008.
169. Seminar, Novozymes, Davis, CA, May 12, 2008.
170. Seminar, Harvard University Medical School, Department of Microbiology, May 27, 2008.
171. Presentation, Royal Society discussion on Synthetic Biology, London, UK, June 2, 2008.
172. Presentation, Burrill & Company, San Francisco, CA, June 10, 2008.
173. Presentation, CITRIS-Copenhagen Research Conference on Climate and Energy, Copenhagen, Denmark, June 18, 2008.
174. Presentation, 4th European Plant Science Organization Conference, Cote d'Azur, France, June 26, 2008.
175. Presentation, Gordon Research Conference on Enzymes, Coenzymes, and Metabolic Pathways, location, July 12, 2008.
176. Presentation, 13th Annual Human Genome Meeting: Genomics and the Future of Medicine, Hyderabad, India, September 28-30, 2008.
177.
178. Keynote Address: "Synthetic biology in pursuit of inexpensive, effective, anti-malarial drugs," EPSRC Centre for Synthetic Biology and Innovation, Imperial College, London, UK, May 12, 2009.

Workshops, Panels, and Short Courses

1. Massachusetts Institute of Technology, Department of Chemical Engineering. August 10-14, 1998. "Metabolic Engineering Short Course."
2. AIChE workshop on Bioinformatics. Houston, TX. March 13-14, 1999.
3. Massachusetts Institute of Technology, Department of Chemical Engineering. August 10-14, 1999. "Metabolic Engineering Short Course."
4. DARPA workshop on Metabolic Engineering. Washington, D.C. March 24 – 26, 2000.
5. Lawrence Berkeley National Laboratory Workshop "Solar to Fuel – Future Challenges and Solutions", Berkeley, CA. March 28-29, 2005.
6. 2005 Genomes to Life Program Workshop, Washington, DC. February 6-14, 2005.
7. Intercollegiate Genetically Engineered Machine Competition (iGEM) 2005 Teacher's Workshop, Boston, MA. May 14-15, 2005.
8. European Science Foundation Exploration Workshop, "Synthetic Biology: Constructing and Deconstructing Life" Arila, Spain. Oct. 13-16, 2005.

Presentations at National or International Meetings

1. J. D. Keasling, A. Joshi, and B. O. Palsson. 1987. "Towards rational design and exploitation of recombinant prokaryotic cells." *194th ACS National Meeting,* New Orleans, LA.
2. J. D. Keasling and B. O. Palsson. 1988. "Dynamics and control of vector replication." *196th ACS National Meeting,* Los Angeles, CA.
3. J. D. Keasling and B. O. Palsson. 1989. "Design in bacterial plasmids." *National AIChE Meeting,* San Francisco, CA.

4. J. D. Keasling, B. O. Palsson, and S. Cooper. 1990. "Cell-cycle-specific F'*lac* plasmid replication: regulation by cell size control of initiation." *European Molecular Biology Organization Meeting on the Bacterial Cell Cycle,* Collonges-La Rouge, France.
5. J. D. Keasling, S. Cooper, and B. O. Palsson. 1990. "Dynamics and control of plasmid replication." *AIChE National Meeting,* Chicago, IL.
6. S. Cooper and J. D. Keasling. 1991. "F plasmid replication: cell-cycle specificity, regulation by cell size control of initiation, and the relationship of different origins of replication to plasmid synthesis." *Human Frontier Science Program Workshop on Regulatory Mechanisms of DNA Replication,* Les Arcs, France.
7. J. D. Keasling and S. Cooper. 1991. "Cell-cycle-specificity, regulation by cell-size control of initiation, and the relationship of different origins of replication to plasmid synthesis." *American Society for Microbiology,* Dallas, TX.
8. S. Cooper and J. D. Keasling. 1991. "Synthesis and regulation of cytoplasm, DNA, cell surface, and plasmid during the bacterial division cycle." *Cold Spring Harbor Symposium on Quantitative Biology,* Cold Spring Harbor, NY.
9. S. Cooper and J. D. Keasling. 1991. "Cell-cycle-specific F plasmid replication during the *Escherichia coli* division cycle: regulation of replication by cell size control of initiation." *Gordon Conference on Extrachromosomal Elements.*
10. J. D. Keasling, S. Cooper, and B. O. Palsson. 1991. "Dynamics and Control of Bacterial Plasmid Replication." *AIChE National Meeting,* Los Angeles, CA.
11. J. D. Keasling, B. O. Palsson, and S. Cooper. 1992. "Plasmid Replication during the Cell Cycle." *Keystone Symposium on Molecular Mechanisms in DNA Replication and Recombination,* Taos, NM.
12. J. D. Keasling, L. Bertsch, A. Kornberg. 1993. "Guanosine pentaphosphate phosphohydrolase of *Escherichia coli* is a long-chain polyphosphatase." *205th ACS National Meeting,* Denver, CO.
13. J. D. Keasling, S. T. Sharfstein, B. Deaton, G. Hupf. 1993. "Engineering and phosphate and energy metabolism in micro-organisms." *Biochemical Engineering VIII,* Princeton, NJ.
14. D. G. Bolesch and J. D. Keasling. 1993. "Anaerobic bioremediation of TCE contamination in groundwater." *Zeneca Process Technology Conference, Leeds, UK.*
15. S. T. Sharfstein, B. Deaton, J. D. Keasling. 1993. "Engineering and phosphate and energy metabolism in micro-organisms." *207th American Chemical Society National Meeting,* San Diego, CA
16. J. D. Keasling, H. Kuo, and G. Vahanian. 1994. "A probabilistic representation of the *Escherichia coli* cell cycle." *AIChE National Meeting,* San Francisco, CA.
17. S. T. Sharfstein, S. J. Van Dien and J. D. Keasling. 1994. "Engineering and phosphate and energy metabolism in micro-organisms." *AIChE National Meeting,* San Francisco, CA.
18. G. A. Hupf, N. Shapiro and J. D. Keasling. 1994. "Manipulation of phosphate and energy metabolism to improve heavy metal resistance and uptake." *AIChE National Meeting,* San Francisco, CA.
19. J. Pramanik and J. D. Keasling. 1994. "Mathematical analysis of fluxes through the metabolic pathways of *Escherichia coli*." *AIChE National Meeting,* San Francisco, CA.
20. R. Pape, P. Jorjani, and J. D. Keasling. 1994. "Design and construction of low-copy plasmids for metabolic engineering of *Escherichia coli*." *AIChE National Meeting,* San Francisco, CA.
21. D. Bolesch and J. D. Keasling. 1994. "Anaerobic bioremediation of chlorinated alkanes." *AIChE National Meeting,* San Francisco, CA.
22. D. Bolesch and J. D. Keasling. 1995. "Anaerobic bioremediation of chlorinated hydrocarbons." *In Situ and On-Site Bioreclamation,* San Diego, CA.
23. G. Hupf and J. D. Keasling. 1995. "Manipulation of phosphate and energy metabolism to improve heavy metal resistance and uptake." *In Situ and On-Site Bioreclamation,* San Diego, CA.

24. J. D. Keasling, S. Van Dien, S. Keyhani, S. Sharfstein. 1995. "Engineering polyphosphate metabolism in bacteria." *Biochemical Engineering VIII,* Davos, Switzerland.
25. P. C. Michels, J. A. Baross, J. D. Keasling, and D. S. Clark. 1995. "Bioremediation potential of newly isolated, metal-tolerant archaea." *Biochemical Engineering VIII,* Davos, Switzerland.
26. J. D. Keasling, S. Van Dien, S. Keyhani, D. Bolesch, and S. Sharfstein. 1995. "Redirection of phosphate and energy metabolism through polyphosphate pathways." *AIChE National Meeting*, Miami Beach, FL.
27. J. D. Keasling, D. Szykowny, and J. Elmen. 1995. "Degradation of aromatic hydrocarbons under denitrifying conditions." *AIChE National Meeting*, Miami Beach, FL.
28. R. Brent Nielsen and J. D. Keasling. 1996. "Anaerobic bioremediation of chlorinated hydrocarbons." Engineering Foundation meeting *Bioremediation of Surface and Subsurface Contamination* in Palm Coast, FL.
29. Joacim Elmen, Dave Szykowny, and J. D. Keasling. 1996. "Degradation of aromatic hydrocarbons under denitrifying conditions." Engineering Foundation meeting *Bioremediation of Surface and Subsurface Contamination* in Palm Coast, FL.
30. J. D. Keasling. 1996. "Metabolic engineering of polyphosphate metabolism in bacteria for phosphate and heavy metal bioremediation." Engineering Foundation meeting *Bioremediation of Surface and Subsurface Contamination* in Palm Coast, FL.
31. Jaya Pramanik and J. D. Keasling. 1996. "A flux-based model of metabolism: effect of biomass requirements and redirected pathways on central metabolism." *211th American Chemical Society National Meeting* in New Orleans, LA.
32. J. D. Keasling. 1996. "Metabolic engineering for bioremediation of inorganic pollutants" *Metabolic Engineering*, Danvers, MA.
33. R. B. Nielsen and J. D. Keasling. 1996. "Kinetic parameter evaluation and modeling of the anaerobic conversion of trichloroethene to ethene." *AIChE National Meeting*, Chicago, IL.
34. N. Eliashberg and J. D. Keasling. 1996. "Simulation of bacterial growth and substrate utilization in a polluted groundwater environment." *AIChE National Meeting*, Chicago, IL.
35. J. Pramanik and J. D. Keasling. 1996. "A flux-based metabolic model for bacteria: study of metabolic regulation and its sensitivity to biomass composition." *AIChE National Meeting*, Chicago, IL.
36. S. J. Van Dien and J. D. Keasling. 1996. "Engineering the polyphosphate levels in *Escherichia coli* and the effects on the phosphate-starvation response." *AIChE National Meeting*, Chicago, IL.
37. J. Pramanik, P. L. Trelstad, and J. D. Keasling. 1996. "Analysis of bioremediation processes using a flux-based metabolic model." *AIChE National Meeting*, Chicago, IL.
38. S. J. Van Dien and J. D. Keasling. 1997. "Engineering the polyphosphate levels in *Escherichia coli*: Effects of energy and phosphate starvation." *ACS National Meeting*, San Francisco, CA.
39. R. B. Nielsen and J. D. Keasling. 1996. "Anaerobic biodegradation of chlorinated hydrocarbons by groundwater microorganisms." *ACS National Meeting*, San Francisco, CA.
40. J. Pramanik, P. L. Trelstad, and J. D. Keasling. 1996. "Analysis of the metabolism of enhanced biological phosphorus removal using a fluxed-based metabolic model." *ACS National Meeting*, San Francisco, CA.
41. J. D. Keasling. 1997. "*In situ* bioremediation of chlorinated and aromatic hydrocarbons in groundwater: application of modern molecular and mathematical tools." *Biochemical Engineering X*, Kananaskis, Canada.
42. J. D. Keasling. 1997. "Development of tools for the metabolic engineering of bacteria." *Biochemical Engineering X*, Kananaskis, Canada.
43. J. D. Keasling, J. Pramanik, J. Benemann. 1997. "Metabolic engineering for hydrogen fermentations." *Biohydrogen '97*, Kona, Hawaii.

44. N. Eliashberg and J. D. Keasling. 1997. "Simulation of spacial heterogeneity development in a mutualistic mixed species biofilm." *AIChE National Meeting*, Los Angeles, CA.
45. R. B. Nielsen and J. D. Keasling. 1997. "Kinetics of anaerobic biodegradation of chlorinated ethenes." *AIChE National Meeting*, Los Angeles, CA.
46. T. A. Carrier and J. D. Keasling. 1997. "Mechanistic modelling of prokaryotic mRNA decay." *AIChE National Meeting*, Los Angeles, CA.
47. S. J. Van Dien and J. D. Keasling. 1997. "Engineering polyphosphate metabolism in *Escherichia coli*." *AIChE National Meeting*, Los Angeles, CA.
48. K. L. Jones and J. D. Keasling. 1997. "Construction, stability, and expression of low-copy vectors derived from the *E. coli* F plasmid." *AIChE National Meeting*, Los Angeles, CA.
49. T. A. Carrier, K. L. Jones, and J. D. Keasling. 1997. "mRNA stability and plasmid copy number effects on gene expression from an inducible promoter system." *AIChE National Meeting*, Los Angeles, CA.
50. R. B. Nielsen and J. D. Keasling. 1998. "Anaerobic degradation of PCE and TCE DNAPLs by groundwater microorganisms." *Remediation of Chlorinated and Recalcitrant Compounds*, Monterey, CA.
51. E. Gilbert, A. Khlebnikov, W. Meyer-Ilse and J.D. Keasling. 1998. "Use of soft X-ray microscopy for analysis of early stage biofilm formation." *Microbial Ecology of Biofilms: Concepts, Tools and Applications. International Association on Water Quality (IAWQ)*, Lake Bluff, IL.
52. K. L. Jones, T. A. Carrier, and J. D. Keasling. 1998. "Plasmid vehicles for long-term, variable gene expression in *Escherichia coli*." *AIChE National Meeting*, Miami Beach, FL.
53. P. L. Trelstad and J. D. Keasling. 1998. "Polyphosphate Metabolism in *Acinetobacter calcoaceticus:* Implications for Enhanced Biological Phosphorus Removal." *AIChE National Meeting*, Miami Beach, FL.
54. R. Brent Nielsen and J. D. Keasling. 1998. "Anaerobic Dechlorination of PCE and TCE DNAPLs by Groundwater Microorganisms." *AIChE National Meeting*, Miami Beach, FL.
55. C. Wang, A. M. Lum, S. C. Ozuna, D. S. Clark, and J. D. Keasling. 1999. Cadmium precipitation by *Escherichia coli* producing cysteine desulfhydrase." *ACS National Meeting*, Anaheim, CA.
56. R. Brent Nielsen and J. D. Keasling, 1999. "Identification of organisms present in a TCE-degrading consortium." *ACS National Meeting*, Anaheim, CA.
57. A. Khlebnikov, O. Risa, and J. D. Keasling. 1999. "Gene expression in a decoupled autocatalytic system under control of inducible promoters." *American Society for Microbiology General Meeting*, Chicago, IL.
58. E. Gilbert, A. Khlebnikov, and J. D. Keasling. 1999. "Dual-GFP labeling of cells in biofilms." *American Society for Microbiology General Meeting*, Chicago, IL.
59. S-W. Bang, D. S. Clark, and J. D. Keasling. 1999. "Precipitation of heavy metals by expression of thiosulfate reductase." *American Society for Microbiology General Meeting*, Chicago, IL.
60. C. Wang, S. C. Ozuna, D. S. Clark, and J. D. Keasling. 1999. "Metabolic engineering of microorganisms to precipitate cadmium wastes." *AIChE National Meeting*, Dallas, TX.
61. A. W. Walker and J. D. Keasling. 1999. "Metabolic engineering of bacteria for the environment: the controlled degradation of parathion." *AIChE National Meeting*, Dallas, TX.
62. P. L. Trelstad, D. Hong, and J. D. Keasling. 1999. "Understanding of the metabolism of enhanced biological phosphorus removal." *AIChE National Meeting*, Dallas, TX.
63. C. D. Smolke, T. A. Carrier, and J. D. Keasling. 1999. "Engineering single and multiple gene expression through mRNA stability control." *AIChE National Meeting*, Dallas, TX.
64. S. Reichmuth, J. D. Keasling, and H. W. Blanch. 1999. "Biodesulfurization of dibenzothiophene in *Escherichia coli* is enhanced by expression of a *Vibrio harveyi* oxidoreductase gene." *AIChE National Meeting*, Dallas, TX.

65. S.W. Kim, K.L. Jones, and J. D. Keasling. 2000. "Expression of 1-deoxy-D-xylulose-5-phosphate synthase in *Escherichia coli* Enhances Lycopene Production". *American Society for Microbiology General Meeting*, Los Angeles, CA.
66. S. E Cowan, E. S. Gilbert, A. Khlebnikov and J. D. Keasling. 1999. "Dual labeling with green fluorescent proteins for confocal microscopy." *IAWQ/IWA Conference on Biofilm Systems, International Association on Water Quality*, New York, NY.
67. K. D. McMahon, M. A. Dojka, N. R. Pace, J. D. Keasling, and D. Jenkins. 1999. "Microbial Community Structure of Laboratory Activated Sludge Performing Enhanced Biological Phosphorus Removal." *American Society for Microbiology General Meeting*. Chicago, IL.
68. E. S. Gilbert and J. D. Keasling. 2000. "Degradation of parathion by a dual-species biofilm consortium." *American Society for Microbiology General Meeting*. Los Angeles, CA.
69. A. Khlebnikov, T. Skaug and J. D. Keasling. 2000. "Elimination of all-or-none gene expression by independent expression of the arabinose transport gene." *American Society for Microbiology General Meeting*, Los Angeles, CA.
70. C. D. Smolke and J. D. Keasling. 2000. "Coordinated, differential expression of multiple genes through directed mRNA cleavage and stabilization by secondary structures." *American Society for Microbiology General Meeting*, Los Angeles, CA.
71. I. Aldor and J. D. Keasling. 2000. "Metabolic engineering of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production in recombinant *Salmonella typhimurium*." *American Chemical Society National Meeting*, San Francisco, CA.
72. E. S. Gilbert and J. D. Keasling. 2000. "Degradation of parathion by a dual-species biofilm consortium." *American Chemical Society National Meeting.* San Francisco, CA.
73. A. Khlebnikov, T. Skaug and J. D. Keasling. 2000. "A regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture." *American Chemical Society National Meeting,* San Francisco, CA.
74. E. S. Gilbert and J. D. Keasling. 2000. "Degradation of parathion by a dual-species biofilm consortium." *Biofilms 2000, American Society of Microbiology*, Big Sky, MT.
75. C. D. Smolke and J. D. Keasling. 2000. "Engineering mRNA stabilizing elements to achieve coordinated, differential expression of two genes." *FASEB Summer Conference in Post-Transcriptional Control of Gene Expression*, Copper Mountain, CO.
76. I. Aldor and J. D. Keasling. 2000. "Metabolic engineering of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production in recombinant *Salmonella typhimurium*." *International Symposium on Biological Polyesters*, Cambridge, MA.
77. K. D. McMahon, N. R. Pace, J. D. Keasling, and D. Jenkins. 2000. "Microbial community structure of activated sludge performing enhanced biological phosphorus removal." *California Water Environment Association Annual Conference,* Sacramento, CA.
78. C. D. Smolke and J. D. Keasling. 2000. "Engineering mRNA stabilizing /destabilizing elements to achieve coordinated differential expression of two genes." *AIChE Annual Meeting*, Los Angeles, CA.
79. A. W. Walker, S. K. Tehara and J. D. Keasling. 2000. "Metabolic Engineering of Bacteria for the Environment: The Degradation of Parathion." *American Institute of Chemical Engineers,* Los Angeles, CA.
80. D.S. Reichmuth, H.W. Blanch and J. D. Keasling. 2000. "Biodesulfurization of dibenzothiophene in *Escherichia coli* is enhanced by expression of a *Vibrio harveyi* Oxidoreductase Gene." *California Catalysis Society Annual Meeting,* Richmond, CA.
81. A. W. Walker, S. K. Tehara and J. D. Keasling. 2001. "Metabolic Engineering of Bacteria for the Environment: The Degradation of Parathion and Paraoxon." *Bioengineering XII,* Sonoma, CA.
82. S.K. Tehara and J.D. Keasling. 2001. "Isolation of a Novel Phosphodiesterase for Biodegradation of Organophosphates." *American Chemical Society*, San Diego, CA.
83. D. S. Reichmuth, J. L. Hittle, H. W. Blanch, and J. D. Keasling. 2001. "Metabolic Engineering of the Dibenzothiophene Biodesulfurization Process." *Biochemical Engineering XII,* Sonoma, CA.

84. G. Y. Wang and J. D. Keasling. 2001. "Isolation and characterization of two key regulatory genes involved in isoprenoid biosynthesis of *Aspergillus nidulans*." *Twenty-First Fungal Genetics Conference*, Pacific Grove, CA.
85. N. L. Goeden, J. D. Keasling, and S. J. Muller. 2001. "Bacterial Expression of a Self-Assembling Amphiphilic Protein Polymer." *AICHE National Meeting*, Reno, NV.
86. N. L. Goeden, J. D. Keasling, and S. J. Muller. 2001. "Bacterial expression of a poly(L-leucylglutamic acid) fusion protein for use in studying structure-property relationships of disordered copolymers." *ACS National Meeting*, San Diego, CA.
87. C. D. Smolke and J. D. Keasling. 2001. "Effects of gene copy number and steady-state mRNA levels on the relative expression levels of two genes in a novel operon." *American Chemical Society National Meeting*, San Diego, CA.
88. C. D. Smolke and J. D. Keasling. 2001. "Effects of gene copy number and steady-state mRNA levels on the relative expression levels of two genes in a novel operon." *American Society for Microbiology General Meeting*, Orlando, FL.
89. V. J. J. Martin, Y. Yoshikuni, and J. D. Keasling. 2001. "A study of the *in vivo* synthesis of plant sesquiterpenes by *Escherichia coli*." *Society for Industrial Microbiology Annual Meeting*, St. Louis, Missouri.
90. K. D. McMahon, D. Jenkins, J. D. Keasling. 2001. "Polyphosphate kinase genes from activated sludge carrying out enhanced biological phosphorus removal." *Water Environment Federation 74th Annual Conference and Exposition (WEFTEC)*, Atlanta, GA.
91. K. D. McMahon, J. D. Keasling, D. Jenkins. 2001. "Polyphosphate kinase genes from activated sludge carrying out enhanced biological phosphorus removal." *International Association for Water Quality 3rd International Specialized Conference on Microorganisms in Activated Sludge and Biofilm Processes*. Rome, Italy.
92. K. D. McMahon, D. Jenkins, J. D. Keasling. 2001. "Polyphosphate kinase genes from activated sludge carrying out enhanced biological phosphorus removal." *101st General Meeting of the American Society for* Microbiology, Orlando, FL.
93. C. D. Smolke and J. D. Keasling. 2001. "Effects of gene copy number and steady-state mRNA levels on the relative expression levels of two genes in a novel operon." *Biochemical Engineering XII*, Rohnert Park, CA.
94. C. D. Smolke, B. Pfleger, and J. D. Keasling, J. D. 2001. "Rational and random design strategies for controlling heterologous protein production from novel operon systems in *E. coli*." *American Institute of Chemical Engineers Annual Meeting*, Reno, NV.
95. N. L. Goeden, J. D. Keasling, and S. J. Muller. 2002. "Microbial Production of a Self-Assembling Amphiphilic Protein Polymer." *American Chemical Society National Meeting*, Orlando, FL.
96. Brian Pfleger, Christina Smolke, and Jay Keasling. 2002. "Engineering mRNA Stability." *Annual Meeting of the Society for Industrial Microbiology*. Philadelphia, PA.
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Docket No.: 066662-0092 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Customer Number: 41552
Palsson, Bernhard	:	Confirmation Number: 1729
Application No.: 09/923,870	:	Group Art Unit: 1631
Filed: August 06, 2001	:	Examiner: Negin, Russell Scott

For: METHODS FOR IDENTIFYING DRUG TARGETS BASED ON GENOMIC SEQUENCE DATA

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Bernhard O. Palsson, declare as follows:

1. I am a Professor in the Department of Bioengineering at the University of California, San Diego (UCSD), San Diego, California. I am also an Adjunct Professor of Medicine at UCSD. I have held the former positions since joining UCSD in 1995, and the latter since 1998. I previously served on the faculty at the University of Michigan from 1984-1995.

2. I obtained a Bachelors of Science majoring in chemical engineering in 1979 from the University of Kansas, and earned my Ph.D. from the University of Wisconsin-Madison in Chemical Engineering in 1984. A copy of my curriculum vitae and a list of publications is attached as Exhibit 1.

3. I am an inventor or co-inventor on at least 35 U.S. Patents and a founder or co-founder of four life science companies. I co-founded Aastrom Biosciences, a public company that focuses on process technologies and devices for cell therapy applications, in 1989 and served as the Vice President of Developmental Research 1994-1995. I am the founder of

EXHIBIT B

Oncosis, a company focused on the purging of occult tumor cells in autologous bone marrow renamed Cyntellect and is focused on building research instrumentation for cell biology.

4. I also am a founder of Genomatica, Inc., which was incorporated in 1998 and is the exclusive licensee of the above-identified application. From 1998 to 2002 I served as the Company's Chief Executive Officer. Currently, I am the chair of Genomatica's Scientific Advisory Board.

5. I am named as the sole inventor on the above-identified patent application, U.S. patent application serial no. 09/923,870.

6. I have read the Office Action mailed December 18, 2008, and am very familiar with the prosecution history of this application. I understand that the claimed invention stands rejected for obviousness over the combination of references to Pramanik et al., *Biotech. and Bioengineering* 56:398-421 (1997) in view of Blattner et al., *Science* 277:1453-69 (1997) and in view of Kunst et al., *Rev. in Microbiol.* 142:905-12 (1991). The Examiner appears to equate the metabolic model described in Pramanik et al. with that described in the subject application and then combines it with two genome sequencing papers (Blattner et al. and Kunst et al. reporting the sequencing of *E. coli* and *B. subtilis* genomes, respectively) to conclude obviousness.

7. It is my opinion that the claimed invention is not obvious over Pramanik et al., Blattner et al. and Kunst et al. because (1) the use of genomic data would not have been expected to produce a predictable model, and (2) the opinions of respected colleagues in the field of metabolic modeling sincerely doubted that such a model would work, even after its publication.

8. One skilled in the art would not have been motivated to use genomic data to produce a stoichiometric matrix because the predictability of an *in silico* model relies on accuracy of the input data. Genome annotation is both incomplete and contains possible errors in the functional annotation of genes. Organism metabolism and reconstructing their metabolic networks from experimental data is scientifically very complex. At the time the invention was made, the metabolic network of a simple organism was known to have hundreds of reactions, substrates, products, co-factors and regulatory interventions and is dependent on many other events occurring within a cell. The predictability of a metabolic model depended on the

accuracy of this complex set of reactions and events. Therefore, metabolic models prior to my discovery employed either known kinetic or biochemical data of known reactions or events in order to ensure the most accurate model as possible.

9. It was incomprehensible to those in the field of metabolic modeling and metabolic engineering to incorporate reactions based on putative gene sequence homology without knowledge that the reaction existed in the modeled organism and without any known kinetic or biochemical information because incorrect deductions would decrease, rather than increase, the predictive capability of the resultant metabolic model. Therefore, those in the field would have discouraged incorporating putative metabolic reactions into an *in silico* model based on gene sequence homology to different organism because it could have led to incorporating erroneous reactions and a loss of a model's accuracy.

10. The unexpected nature of my discovery that genomic data can be incorporated into a model to construct a stoichiometric matrix without other knowledge is borne out by the disbelief of respected colleagues in the field at the time the invention was made. In addition to the concerns about the accuracy of the genomic data, my colleagues doubted that a metabolic reconstruction containing information about only 15% of the identified genes in *E. coli* and no regulatory information would be able to lead to meaningful computations about physiological functions.

11. Professor James E. Bailey, a respected and prestigious scientist in the field of metabolic engineering, is one such colleague who, on the one hand, found the success of my approach hard believe, but on the other hand if the model worked as purported, characterized my genomic-flux balance approach a "very major advance," a "breakthrough."

12. Professor Bailey passed away on May 9, 2001. At the time of my invention, Professor Bailey was a professor in the Institute of Biotechnology, ETH Zurich, Swiss Federal Institute of Technology in Zurich, Switzerland. Professor Bailey held a great deal of stature and respect in the field of metabolic engineering. Among Professor Bailey's many accomplishments include the foundational paper on Metabolic Engineering ('Towards a Science of Metabolic Engineering' Science 252:1688-1675, 1991, attached as Exhibit 2) and the publication of the standard text book in biochemical engineering (J.E. Bailey and D.F. Ollis, BIOCHEMCIAL

ENGINEERING FUNDAMENTALS, 2nd edition 1986, 984 pages total, McGraw-Hill, New York). Publications in memory of Professor Bailey summarizing his accomplishments, scientific career and stature in the field of metabolic engineering are attached as Exhibits 3 and 4.

13. Attached as Exhibit 5 is a letter from Professor Bailey to me dated July 8, 1999, commenting on my publication describing the robustness of an *E. coli* metabolic model that used genomic data in its construction. A copy of the publication that precipitated Professor Bailey's letter is attached as Exhibit 6 (Edwards and Palsson, *Biotechnol. Prog.* 16:927-39 (2000).

14. With some tenacity Professor Bailey questions the operability of a genomic-flux balance model and the case studies reported in Exhibit 4, finding them "hard[] . . . to understand" (paragraph 2) and raising a number of questions regarding how the genomic model arrives at rates from stoichiometry (paragraph 3). Professor Bailey also questions the inclusion of 47 enzymes in addition to those implied by the genome sequences (paragraph 4). These steps were added based on biochemical information alone.

15. In the concluding paragraph Professor Bailey shows his very real skepticism and reservation by explicitly questioning whether the genomic model is correct or whether it works. Conversely, if the model operates as described Professor Bailey characterizes the discovery as a breakthrough. This skepticism and acknowledgement that the discovery is a breakthrough in the field of metabolic engineering are clear when Professor Bailey states:

> This method, if it is correct and if it works, is a very major advance, because it give a formalism for determining growth rates and pathway rates without any knowledge of any kinetics. I must say it is a little hard to believe that this can be done, but maybe you have made a breakthrough. *Id.* (emphasis added).

As an opinion leader in the field, it is fair to say that Professor Bailey's skepticism characterized the attitudes of my colleagues at the time.

16. Since my initial publication of a genomic-flux balance metabolic model and the above interaction with Professor Bailey there have now been probably more than 100 subsequent publications from my laboratory and from the work of others in the field documenting the operability and predictive power of this type of metabolic model. This discovery was unexpected because we found that we could incorporate metabolic reactions based on genomic

deductions and the model was able to accurately predict metabolic capabilities of the modeled organism. As would be characterized by Professor Bailey now that there are several hundred subsequent publications to quell his initial skepticism, this discovery was a very major advance or breakthrough in the field. For example, for *E. coli* alone, about 70 such scientific studies were reviewed in June 2008 (Nature Biotechnology, 26: 659-667, 2008), attached as Exhibit 7. Notable predictions by the genomically-based reconstruction were the computation of the effects of gene knock-outs (Edwards and Palsson, *Proc. Nat'l Acad. Sci.USA*, 97:5528-33, (2000) (Exhibit 8); Covert et al., *Nature,* 429:92-96 (2004) (Exhibit 9)) and the outcomes of adaptive evolution (Ibarra et al., *Nature,* 420:186-189 (2002) (Exhibit 10)). These predictions were unexpected and widely noticed by the scientific community.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Bernhard Palsson June 16th 2009

SDO 150

CURRICULUM VITAE

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EDUCATION

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- University of Kansas, BS in Chemical Engineering, 1977-1979,
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- University of Kansas, Graduate Courses in Biochemistry, 1978-1979.
- University of Wisconsin, Ph.D. in Chemical Engineering, 1979-1984,
  Dissertation: Mathematical Modeling of Dynamics and Control in Metabolic Networks. Minor: Mathematics and Biochemistry.

PROFESSIONAL EXPERIENCE

- *Teaching assistant*:
  University of Wisconsin, 1980 - 1984 in process design and in process control.

- *Research assistant*:
  University of Iceland, 1976-1977,
  a) fatty acid oxidizing enzymes in cardiac muscle and
  b) statistical analysis of biochemical data
  University of Kansas, 1978-1979,
  a) cell kinetics, and
  b) diffusion in polymer matrices
  University of Wisconsin, 1980-1984,
  a) economic evaluation of fermentation products as feedstocks for the chemical industries,
  b) development of non-linear control theory, and
  c) mathematical modeling of dynamics and control in metabolic networks
- Assistant Professor, 1984-1990, Department of Chemical Engineering,
  The University of Michigan, Ann Arbor
- Co-Founder, 1989, Ann Arbor Stroma Inc (renamed Aastrom Biosciences Inc)
- Associate Professor, 1990-1995, Department of Chemical Engineering,
  The University of Michigan, Ann Arbor
- George Granger Brown Associate Professor of Chemical Engineering, 1991-1995, The University of Michigan, Ann Arbor
- Vice-President for Developmental Research, 1994-1995,
  Aastrom Biosciences, Inc., Ann Arbor
- Professor, 1995-present, Department of Bioengineering,
  University of California-San Diego
- Visiting Professor, 1996, Department of Process Biotechnology,
  The Technical University of Denmark, Lyngby
- Founder, Chairman, 1997-Present, CEO (Until 2002), Oncosis/Cyntellect,
- Co-Founder (1998), Chairman (Until 2002), CEO (Until 2000), Iceland Genomics Corporation

- Adjunct Professor of Medicine, UCSD, 1998-Present
- Co-Founder (1999), Chairman, Genomatica, 1999-2001
- Hougen Professor, University of Wisconsin, 2000.
- Galetti Professor of Bioengineering, UCSD, 2004-Present
- Faculty Member, Keio University, Tokyo, 2005-Present

SCHOLARSHIPS, AWARDS, AND DISTINCTIONS

Graduation from the University of Kansas with highest distinction, 1979
Inclusion in Who's Who among students in American Colleges and Universities, 1979
Award for outstanding undergraduate research, 1979
Fellow of the Institute of International Education, 1977-1979
Fellow of Rotary International, 1979-1980
Experiment Station Research Assistantship in Chemical Engineering, 1982-1983
NATO Fellow, 1984
FIRST award from the NIH, 1987-1992
George Granger Brown Professorship, 1991-1995
Chemical Engineering Research Award, University of Michigan, 1993
Fellow of the American Institute for Medical and Biological Engineering, 1995
Fulbright Fellowship and Ib Henriksen Fellow, 1996
Hougen Professorship, University of Wisconsin, Madison, 2000.
Lindbergh-Carrel Prize, 2001
Bayer Lectures Honoree, 2001
FPB/AIChE Award, 2002
NIH Computational Approaches to Biological Systems Seminar Series, 2003
Keynote Address, ESCAT Meeting, Spain, May, 2003
Keynote Address, Mass Spectrometry In Systems Biology, Santa Fe, NM, February 2004
Plenary Talk, ISAC International Congress, Montpelier, France, May, 2004
Keynote Address, 5th International Conference on Systems Biology, Heidelberg, Germany, October, 2004.
Keynote Address, IBC Cell Line Development and Engineeirng Conference, St. Louis, November, 2005.
Election to National Academy of Engineering, February, 2006.
UCSD Chancellor's Associates Faculty Award for Excellence in research in Science and Engineering, 2006
Selected as one of 47 most influential labs for technology development over 10 years, *Nature Biotechnology*, March, 2006.
Plenary Talk, IECA 2006, Jeju, Korea, October, 2006.
Plenary Talk, Summit on Systems Biology, Richmond, Virginia, June 2007.
Plenary Talk, Metabolomics Society 3rd Annual Conference, Manchester, UK, June, 2007.
Richard S.H. Mah Lecture, Northwestern University, October, 2007.
Plenary Lecture, Metabolic Engineering VII, Puerto Vallarta, September, 2008.
Keynote Address, IBC Discovery 2 Diagnostics Conference, San Diego, October, 2008.
The Ernst W. Bertner Memorial Award, 2008.
Honorary Doctorate Degree, Chalmers University of Technology, May, 2009.

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EDITORIAL ACTIVITIES

1. Special Editor, with Jeffrey A. Hubbell and E.T. Papoutsakis, of Tissue Engineering & Cell Therapies: I and II, special issues of *Biotechnology and Bioengineering*, 43:7 and 43:8, March 25 and April 5, 1994, John Wiley & Sons, Inc., Publishers.
2. Editorial Board, *Tissue Engineering*, Charles A. Vacanti and Antonios G. Mikos, editors, Mary Ann Liebert, Inc., Publishers. (1994 to 2000)
3. Board of Associate Editors, *Mathematical Problems in Engineering*: Problems, Theories, and Applications, V. Lakshmikantham and S.M. Meerkov, Editors, Gordon & Breach Publishing Co. (1994 to 2000)
4. Editorial Board, *Annals of Biomedical Engineering*, James B. Bassingthwaighte and Daniel A. Hammer, Editors (1995 to present)
5. Section editor for Tissue Engineering, with Jeffery A. Hubbell, *The Biomedical Engineering Handbook*, Editor-in-Chief Joseph D. Bronzino, CRC Press, Boca Raton (1995).
6. Editorial Board, *Biotechnology and Bioengineering*, Douglas Clark Editor-in-Chief, Wiley and Sons (1996-present)
7. Editorial Board, *Metabolic Engineering*, G. Stephanopoulos, M. Yarmush, and A. Sinskey, Editors, Academic Press.
8. Advisory Editorial Board, *Nature Molecular Systems Biology*, (2004-Present).
9. Editorial Board, *Journal of Bacteriology*, (2004-Present).
10. Editorial Board, *Journal of Biological Chemistry* (2007-Present).

PROPOSALS ADMINISTERED

TITLE	SOURCE	AMOUNT	CO-PI/PERIOD
Mathematical Modeling of Anaerobic Digestion Dynamics	Michigan Biotechnology Institute	$22,500	18 months, (1985).
Use of Kinetic Models to Improve Blood Storage	Whitaker Foundation	$82,532	Nov 1985 - Oct 1987
Cell Culture Facility	University of Michigan NIH Biomedical Research Support Grant	$59,500	Professors J. S. Schultz and H. Y. Wang (1986)
Cell Culture Facility	NSF equipment grant	$21,300	H.Y. Wang Co-PI (1986)
Purchase of a Spectrofluorometer	University of Michigan NIH Biomedical Research Support Grant	$14,000	Professor J. S. Schultz (1987)
Cellular Bioengineering	From the Presidential Initiatives Fund at the University of Michigan	$90,000/Yr.	Prof. M. Savageau (PI), with Profs M.E. Meyerhoff and A. R. Midgley, June 1987 - May 1990
Metabolic Dynamics in the Red Cell	FIRST award from NIH	$90,000/Yr.	Sept 1987 - Aug 1992
Efficient Monoclonal Antibody Production	NSF Biotechnology Cluster Grant	$803,520	Professors M. A. Savageau and A. R. Midgley, Sept 1987 - Sept 1990,
Life Support Systems via the Use of Cell Culture	CAMRSS, a NASA funded CCDS center	$150,000	Prof. P. Kaufman and Dr. T. Huard, Feb 1988 - Jan 1989
Construction of a High-efficiency Ex vivo Bone Marrow	NSF Tissue Engineering Initiative	$50,000	Drs. S. Emerson (PI) and M. Clarke in Internal Medicine, Sept 88 to Aug 89
Gravitropic Response Mechanism in Cereal Grass Shoots	NASA	$55,000	Prof. P. Kaufman (PI) 2/89-6/89
Development of an Ex vivo Bone Marrow System	Hambrecht & Quist	$300,000	S.G Emerson (PI) and M. Clarke 4/89 - 10/89
Development of a Photo-bioreactor and Green Plant Cell Lines for CELSS	CAMRSS, a NASA funded CCDS Center	$250,000	Professor P. Kaufman, Feb 1989 - Sept 1989
Development of an Ex vivo Bone Marrow System	Ann Arbor Stromal Inc.,	$900,000	S. Emerson (PI) and M. Clarke 8/89-4/92
Development of an Algal Photo-	CAMRSS, a NASA-funded	$120,000	Nov. 1989-Sept 1990.

bioreactor	CCDS center		
Establishment of a Digital Imaging Facility	BSRG/NIH	$27,000	Prof. J.J. Linderman (PI) April 1990 - March 1991.
Efficient Monoclonal Antibody Production	NSF Biotechnology Cluster Grant	$664,098	Prof. M. E. Meyerhoff, Nov 1990 - Oct 1993
Photo-bioreactor Engineering: Light Delivery and Algal Growth and Gas Exchange Rates	NASA/Headquarters	$960,713	Prof. T. L. Killeen, Jan, 1991 to Jan, 1994.
Development of an ex vivo Retroviral Infection System	Aastrom Biosciences Inc.	$30,000	Post-doctoral Fellowship 1/14/91 - 10/13/92.
Development of a Hematopoietic Bioreactor System	Aastrom Biosciences Inc.	$45,000	Post-doctoral Fellowship 5/1/92 - 10/31/93.
Ex vivo Growth and Manipulation of Human Hematopoietic Cells - Hematopoietic Cell Expansion Bioreactor Design and Retroviral Gene Transfer	Aastrom Biosciences Inc.	$532,259	3/1/92 - 6/30/93.
Shear Sensitivities of Human Bone Marrow Cultures	NASA Headquarters	$315,000	12/1/92-12/1/93.
Hematopoietic Bioreactor Design with Applications to Gene Therapy	Aastrom Biosciences, Inc.	$344,001	7/1/93 - 12/31/94.
Biological Determinants of Photobioreactor Design	Department of Energy	$99,898	9/1/93 - 8/31/95.
Hemtaopoietic Cell Expansion System	NIH SBIR Phase II Award	$520,000	3/1/95 - 2/29/96.
Biochemical Engineering: Genomatics and Whole Cell Simulators	UC Biotechnology Program	$80,000	7/1/96-6/30/98
Shear Sensitivities of Human Bone Marrow Cultures	NASA Biotechnology Program	$190,000	10/1/95-12/1/97.
Stem Cell Motility	The Stern Foundation	$50,000	11/1/97-10/30/98
Hematopoietic Stem Cell Motility	National Institutes of Health, R01 HL59234	$152,207 annual direct costs	Jan 1, 1998 to Dec 31 2001
Genomically Based Models for Antibiotic Development	National Institutes of Health, R01 GM57089	$123,000 annual direct costs	7/1/98 - 6/30/01
Mechanisms of Stem Cell Migration	National Institutes of Health, R01 HL 60398	$200,000 annual direct costs	7/10/98 to 7/9/02
In Silico Analysis of the Escherichia Coli Metabolic Genotype and the Construction of Selected Isogenic Strains	National Science Foundation/Department of Defense, BES-9814092	$88,388/Yr.	3/1/99-2/28/02
Computational Infrastructure for Engineering Microorganisms	National Science Foundation/KDI, SBR-9873384	$297,218	10/1/98-9/30/00
Genome-scale *in silico* Model for *E. coli*	National Institutes of Health, R01 GM057089	$250,000	8/1/98-4/30/11
Tissue Engineering	Whitaker Foundation/Teaching Materials Project	$89,962	11/1/99-1/1/03
Kinetic and Regulatory Constraints on Metabolism	National Science Foundation/BES-0120363	$469,934	9/1/01-8/31/03
Testable *in silico* Hypotheses for *E. coli* Growth	National Institutes of Health, RO1 GM62791	$432,254 annual direct costs	4/01/01-5/31/10
Genomically Based Models for Antibiotic Development (Renewal)	National Institutes of Health, R01 GM57089	$250,000 annual direct costs	4/1/03 - 3/31/08
Systems Biology and Bioengineering	Whitaker Foundation/Teaching Materials Project	$84,713	4/1/03-11/30/04
Network Based Analysis of Kinetics and Regulation	National Institutes of Health/R01 GM68837	$225,000 annual direct costs	7/15/03-6/30/07
Reconstruction and Simulation of	National Science	$432,697	10/1/03-9/30/06

Genome-Scale Regulatory Networks	Foundation/BES-0331342		
A Genome-Scale Regulated Metabolic Model of Yeast	National Institutes of Health, RO1 GM071808	$446,123 annual direct costs	7/01/04-6/30/09
Analysis of the Genetic Potential and Gene Expression of Microbial Communities Involved in the in situ Bioremediation of Uranium and Harvesting Electrical Energy from Organic Matter	DOE, 03-003721 G 00	$254,750	9/1/06-1/31/09
Systems-Level Understanding of Hydrogen Production by *Thermotoga maritima*.	DOE, DE-PS02-08ER08-12	$506,489	9/15/08-9/14/11
A Systems Biology Program to Study Infectious Microorganisms in Taiwan	NHRI (Taiwan)	$1,052,904	7/1/08-8/31/11

INVITED TALKS PRESENTED AT MEETINGS:

1. B. O. Palsson "Making Mathematical Descriptions of Metabolic Reaction Networks Manageable" Presented at the *3rd Henry Goldberg Workshop* on "Simulation and Modeling of the Cardiac System: from Cellular Activation to Muscular Activity", March 31 - April 2, 1986, Rutgers University, New Brunswick, NJ.
2. O. Palsson, A. Joshi, and S. S. Ozturk, "Reducing Complexity in Metabolic Networks: Making Metabolic Meshes Manageable", *FASEB meeting,* St. Louis, MO, April 14-18, 1986.
3. B. O. Palsson, I-der Lee, and A. Joshi, "A Comprehensive Computer Model of Human Red Cell Metabolism," *International Symposium on Mathematical Models of Cellular Processes*, Holzhau, GDR, November 19-23, 1989
4. B. O. Palsson, S. Emerson, M. Clarke, R. Schwartz, and J. Caldwell, "Reconstitution of a Functioning Bone Marrow," *The 1989 International Chemical Congress of Pacific Basin Societies*, Symposium on Cell Culturing, Honolulu, Hawaii, December 17-22, 1989
5. B. O. Palsson, "Metabolic Modeling, Design and Operation of Continuous Perfusion Hematopoietic Cultures," *UCLA Symposium on Tissue Engineering*, Keystone CO, April 5-12, 1990
6. Minoo Javanmardian and B. O. Palsson, "Can Biotechnology Help with Global Warming?," *American Chemical Society 200th National Meeting*, Washington, D.C., August 26-31, 1990
7. J. D Keasling, B. O. Palsson, and S. Cooper, "Cell-cycle-specific F' lac Plasmid Replication: Regulation by Cell Size Control of Initiation," *European Molecular Biology Organization Meeting on the Bacterial Cell Cycle*, Collouges-LaRouge, France, September 30 - October 5, 1990.
8. B. O. Palsson, "Hematopoietic Bioreactor Systems," Engineering Foundation Conference - *Cell Culture Engineering III: Tissue Engineering*, Palm Coast, Florida, February 2 - 7, 1992.
9. A. Peng and C. G. Lee, "Two Novel Bioreactor Systems for the Cultivation of Human Bone Marrow and for Growing Algae Photoautotrophically," *SIM/CSM Joint Meeting - Symposia on Novel Bioreactors*, Toronto, Canada, August 1 - 6, 1993.
10. B. O. Palsson, "Hematopoietic Tissue Engineering: From Basic Principles to Clinical Practice," *IFMBE 1st International Conference on Cellular Engineering*, Stoke-on-Trent, UK, September 12 - 15, 1993.
11. B. O. Palsson, "Cell and Tissue Engineering: An Emerging Discipline?" *N + N Meeting on Cellular Engineering,* USA and UK, Chester, UK, September 15-17, 1993.
12. B. O. Palsson, "Tissue Engineering and Differentiating Cell Types," *Frontiers in Bioprocessing III*, Boulder, CO, September 19-23, 1993.
13. B. O. Palsson, "Shear Sensitivities of Human Bone Marrow Culture," *Investigators' Meeting,* NASA/Johnson Space Center, Houston, TX October 15-16, 1993.
14. C-G. Lee and B.O. Palsson, "Design and Performance of an LED-Based Algal Photobioreactor," *International Winter Meeting of the American Society of Agricultural Engineers* (ASAE) Chicago, IL, December 14-17, 1993.

15. B. O. Palsson, "Hematopoietic Perfusion Bioreactors: Scientific and Clinical Utility," Keystone Symposia on Molecular and Cellular Biology: *Tissue Engineering*, Taos, New Mexico, February 20-26, 1994
16. B. O. Palsson, "Expansion of Progenitor Cells," *The Seventh International Symposium on Autologous Bone Marrow Transplantation*, Arlington, TX, August 17-20, 1994.
17. B. O. Palsson, "Hematopoietic Tissue Engineering," *ESACT/JAACT Meeting Animal Cell Technology 'Developments towards the 21st Century,'* Veldhoven, The Netherlands, September 12-16, 1994.
18. B. O. Palsson, "Perfusion-Based Bioreactors for the Expansion of Human Bone Marrow, Stem and Progenitor Cells," *BPEC Symposium and Workshop on Bioprocessing Needs for Cell Based Therapies*, MIT, Cambridge, MA, January 18-19, 1995.
19. B. O. Palsson, "Tissue Engineering of Bone Marrow," *National Engineering Forum*, UC San Diego School of Engineering, San Diego, May 4-5, 1995.
20. B. O. Palsson, M.R. Koller and J. Maluta, "Hematopoietic Tissue Engineering Challenges in Producing Clinical Scale Cell Populations," Engineering Foundation *Biochemical Engineering IX: Interdisciplinary Foundations for Creating New Biotechnology,* Davos, Switzerland, May 21-26, 1995.
21. B. O. Palsson, M.R. Koller and J. Maluta, "Hematopoietic Tissue Engineering Challenges in Producing Clinical Scale Cell Populations*," 2nd International Meeting on Cell Engineering*, San Diego, August 1995
22. B. O. Palsson and A. Varma, "Metabolic Flux Balancing: basic concepts, scientific and practical use," *Recent Advances in Fermentation Technology*, San Diego, November 4-7 1995
23. BO Palsson, J. Maluta, CA Peng, RD Armstrong and MR Koller, "Scientific and Clinical Applications of and Ex vivo Hematopoietic Model," *Tissue Engineering*, Keystone Symposia, Taos, NM Jan 1996
24. BO Palsson, AS Chuck and G Huang, "Gene Therapy: The Importance of Random Brownian Motion," *Cell Culture Engineering V*, Engineering Foundation Meetings, San Diego, Jan 1996
25. BO Palsson, AS Chuck and G Huang, "Retroviral Infection is Limited by Random Brownian Motion," *Gene Therapy for Hematopoietic Stem Cells in Genetic Disease and Cancer*, Keystone Symposia, Taos, NM Feb 1996
26. BO Palsson, "Retrovirally-Mediated Gene Transfer is Limited by Random Brownian Motion," 4th International Symposium *on Recent Advances in Hematopoietic Stem Cell Transplantation: Clinical Progress, New Technology, and Gene Therapy.* UCSD/ISHAGE, San Diego, CA, April 11-13, 1996'
27. BO Palsson, "How Should We Approach the 'Engineering' of Metabolic Function," *Recombinant DNA Biotechnology: Focus on Metabolic Engineering*, Engineering Foundation Conferences, Ferncroft Conference Resort, Danvers, MA, October 6-11, 1996
28. BO Palsson, "Modeling Challenges in Tissue Engineering and Complex Systems," *Modeling in Biochemical Engineering*, October 11-12, 1996 at the University of Minnesota, Minneapolis/St. Paul, MN.
29. BO Palsson, "Genetic Circuits," *Chemical Engineering and Living Systems,* in Honor of Professor EN Lightfoot, Nov 9-10, 1996, Madison, WI
30. J Edwards, R Ramakrishna and BO Palsson, "Significant Flexibility Exists in the Core Metabolic Pathways," Soc. for Industrial Microbiology Annual Meeting, Reno, NV, Aug 3-7th, 1997
31. B.O. Palsson "What lies Beyond Bioinformatics?," National Academy of Engineering, Annual Meeting, October 6 to 8, 1997, Washington DC
32. B. O. Palsson "The Importance of Stem Cells in Tissue Engineering: lessons learned from hematopoiesis," Tissue Engineering, Keystone Symposia, Copper Mountain, Jan 1998
33. B. O. Palsson "The Importance of Stem Cells in Tissue Engineering: lessons learned from hematopoiesis," Cell Culture Engineering VI, Engineering Foundation Conference, Pacific Beach, February 8-14, 1998
34. BO Palsson, "What Lies Beyond Bioinformatics?," Bioinformatics Workshop, Sat. April 4, 1998 San Diego Supercomputer Center Auditorium
35. BO. Palsson, "Mechanisms of Stem Cell Migration," 6th International Symposium on Recent Advances in Hematopoietic Stem Cell Transplantation," April 16-18 San Diego, 1998
36. BO. Palsson, "Bioinformatics--Their Role in Modern Scientific Investigation and Cardiac Disease," 6th Antwerp-La Jolla-Kyoto Research Conference on Cardiac Function, La Jolla, April 25-27, 1998

37. BO. Palsson, "Towards Metabolic Phenomics: analysis of genomic data using flux balances," Metabolic Engineering II, Elmau, Germany, Oct 25-30, 1998
38. B.O. Palsson, "Haemophilus influenzae Metabolic Genotype; Its definition, Systems, Characteristics, and Capabilities," AIChE Annual Meeting, Miami Beach, FL November, 1998.
39. B.O. Palsson, "Synthesizing and Characterizing In Silico Bacterial Strains," In Silico Biology Meeting, San Francisco, CA, June 1999.
40. B.O. Palsson, "Building Metabolic Models from Annotated Genes," Biochemical Engineering XI: Molecular Diversity In Discovery and Bioprocessing, Park City, UT, July 1999.
41. B.O. Palsson, "Life on the Edge: using genome-scale *in silico* models of microorganisms to interpret and predict metabolic phenotypes," 11th International Genome Sequencing and Analysis Conference, Miami, FL, September 1999.
42. B.O. Palsson, "What Lies Beyond Bioinformatics?," BMES/EMBS Joint Meeting, Atlanta, GA, October 1999.
43. B.O. Palsson, J.S. Edwards, and R.I. Ibarra, "The generation of experimentally testable hypotheses *in silico* using an *Escherichia coli* metabolic model, 4th Annual Hilton Head Workshop, Hilton Head, SC, February, 2000.
44. B.O. Palsson, "Reconstruction of Metabolic Networks in silico and Formulation of Testable Experimental Hypotheses," Bioinformatics 2000, Elsinore, Denmark, April, 2000.
45. B.O. Palsson, "Living on the edge: E. coli optimizes its growth within governing physico-chemical constraints," In Silico Biology Conference, San Francisco, June, 2000.
46. B.O. Palsson, "Functional Genomics," The Whitaker Foundation Biomedical Engineering Education Summit, Arlington, VA, December, 2000
47. B.O. Palsson, "Models of microorganisms to interpret and predict metabolic phenotypes: basic concepts, scientific and applied uses," NASCRE 1 Conference, Houston, TX January, 2001.
48. B.O. Palsson, "Single and multi cellular communication pathways and their transduction," DOE/NSF Workshop on Biological Information Processing and Systems," Greenville, SC, January, 2001.
49. B.O. Palsson, "The challenges of *in silico* biology," Workshop on Challenges and Opportunities in Data Management, Palo Alto, CA, March, 2001.
50. B.O. Palsson, " Life on the edge: Using genome-scale *in silico* models of microorganisms to interpret and predict metabolic phenotypes," Recovery of Biological Products 10, Cancun, Mexico, June, 2001
51. B.O. Palsson, "Influence of Bioinformatics on Metabolic Engineering," CAB8 Conference, Quebec, Canada, June, 2001.
52. B.O. Palsson, "Do *in silico* models of metabolism represent their *in vivo* counterparts well?" Beyond Genome 2001, San Francisco, June, 2001.
53. B.O. Palsson, "The Phase transition from *in vivo* to *in silico* biology," The Bayer Lectures in Chemical Engineering, Berkeley, September, 2001.
54. B.O. Palsson, "Life on the edge: using genome scale in silico models of microorganisms to interpret and predict metabolic phenotypes," DOE 9th International Conference on Microbial Genomes, Gatlinburg, TN, October, 2001.
55. B.O. Palsson, "Data-Driven Constraints-Based Models in Biology," AIChE Meeting, Reno, NV, November, 2001.
56. B.O. Palsson, "The Phase transition from *in vivo* to *in silico* biology," Syngenta, December, 2001
57. B.O. Palsson, "The Phase transition from *in vivo* to *in silico* biology," CHI Metabolomics Meeting, December, 2001
58. B.O. Palsson, "The phase transition from *in vivo* to *in silico* biology," ETH, Zurich, Switzerland, January, 2002.
59. B.O. Palsson, "The phase transition from *in vivo* to *in silico* biology," Novartis, Basel Switzerland, January, 2002.
60. B.O. Palsson, "Data Driven Constraint-Based Models in Cell Biology," 6th Annual Lake Tahoe Symposium, Granlibakken, Lake Tahoe, CA, January, 2002.
61. B.O. Palsson, "From bioreactor design to genetic circuits, " Lindbergh-Carrel Symposium, Charleston, SC, February, 2002.
62. B.O. Palsson, "Model-Centric Integrated Genomic Databases," Frontiers of Genomics, Madison, Wisconsin, May 16-17, 2002.
63. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models" Cuernevaca, Mexico, May 23-25, 2002.

64. B.O. Palsson, "The New Biotechnology Seminar Series," Campbell and Flores, LLP, June, 2002.
65. B.O. Palsson, "Keynote Speaker," IEEE Computer Society Bioinformatics Conference, Stanford University, August 14-16, 2002.
66. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," Engineering Foundation Conferences, Metabolic Engineering IV, Barga, Italy, October 6-11, 2002.
67. B.O. Palsson, FPB Division Award Talk, "The phase transition from *in vivo* to *in silico* biology," AIChE Meeting, Indianapolis, IN, November 3-5, 2002.
68. B.O. Palsson, "Genome-Scale Models for Prospective Metabolic Engineering," CHI—Metabolic Engineering: Modifying Metabolic Pathways Conference, Raleigh, NC, December 2-3, 2002.
69. B.O. Palsson, "Laser-Enabled High-Throughput High-Content Single-Cell Analysis," IBC—Cell Based Assay and Screening, Philadelphia, PA December 4-6, 2002.
70. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," Harvard University Department of Genetics, December 10, 2002.
71. B.O. Palsson, "Computer-assisted search for new antimicrobials," 2002 CSPA ANTI Special Session, Ft. Lauderdale, FL, December, 2003.
72. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models, ICSB 2002, Stockholm, Sweden, December 13-15, 2002.
73. B.O. Palsson, "A model-driven analysis of expression data for E. coli and for Yeast," EMBO Practical Course, University of Milano-Bicocca, Italy, January 13, 2003.
74. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," Plenary Talk-Enzymes & Biocatalysis for Drug Discovery and Development, San Diego, CA, January 30, 2003.
75. B.O. Palsson, "Genome-scale analysis for new metabolic engineering procedures," National Science Foundation Metabolic Engineering Conference, January 31, 2003.
76. B.O. Palsson, "Bringing Genomes to Life: The Key Role of Genome-scale Computer Models," IRI Fronteirs of Technology 2003 Conference, February 27-28, 2003.
77. B.O. Palsson, "Novel HTC?HTS Technology," BiolT Meeting, Boston, March 26, 2003.
78. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-scale *in silico* Models," NIH Computational Approaches to Biological Systems Seminar Series, March 27, 2003.
79. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-scale *in silico* Models," IBM Research Computational Biology Seminar, March 28, 2003.
80. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-scale *in silico* Models," UCSD Bioinformatics Symposium, March 29, 2003.
81. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-scale *in silico* Models," Plenary Talk-Northwestern University Computational Science and Engineering Spring Symposium, April 3, 2003.
82. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models" Duke Center for Bioinformatics and Computational Biology Conference, May, 2003.
83. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," Keynote Address-ESCAT Meeting, Spain, May, 2003.
84. B.O. Palsson, "Chairperson's Remarks," CHI Beyond Genome 2003, San Diego, June, 2003.
85. B.O. Palsson, "E. coli i2K," IECA 2003, Tsuruoka, Japan, June21-26, 2003.
86. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," The Nyhan Center Planning Meeting, Palo Alto, September, 2003.
87. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," GTL Data Standards Workshop, San Francisco, CA, September, 2003.
88. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," ERATO Kitano Project, Tokyo, Japan, September, 2003.
89. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," Southern California Biotechnology Symposium, Laguna Beach, CA, September, 2003.
90. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," NAE Annual Meeting, Washington, DC, October, 2003.
91. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," International Symposium on New Horizons in Molecular Sciences and Systems: An Integrated Approach, Okinawa, Japan, October, 2003.
92. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models," IBC Systems Biology for Drug Discovery and Development, Boston, MA, October, 2003.
93. B.O. Palsson, "Bringing Genomes to Life: The Use of *in silico* Models,", NIH SysBio SIG Retreat, Washington, DC, November, 2003.

94. B.O. Palsson, "Systems Biology and Genetic Circuits," 2004 Gordon Conference in Molecular Evolution, Ventura, CA, February, 2004.
95. B.O. Palsson, "Metabolomics," Mass Spectrometry in Systems Biology, Keynote Address, Santa Fe, NM, February, 2004.
96. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," Rutgers Collaboratus, XIV, Rutgers University, March, 2004.
97. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," Invited Seminar, Caltech, April, 2004.
98. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," ISAC International Congress, Plenary Talk Montpelier, France, May, 2004.
99. B.O. Palsson, "*E. Coli* 2K: A Chemically and genetically structured framework for relating the virtual and real Gemini," Second International *E. Coli* Alliance Conference on systems Biology, Alberta, Canada, June, 2004.
100. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," the 250th Anniversary of Columbia University—A Symposium for Biomedical Engineering, New York, September, 2004.
101. B.O. Palsson, "Combining Genome-Scale Reconstructions, OptKnock and Adaptive Evolution to Design Production Strains," Metabolic Engineering V, Lake Tahoe, September, 2004.
102. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," The University of Texas, Austin Seminar Series, Austin, September, 2004.
103. B.O. Palsson, "Critical Issues in Systems Biology," IBC Systems Biology Summit, Boston, September, 2004.
104. B.O. Palsson, "Using *in silico* Models of Microorganisms to Elucidate Metabolic Pathways," Arrowhead 2004, Lake Arrowhead, September, 2004.
105. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," BioJapan 2004, Tokyo, September, 2004.
106. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," 2nd International Conference on Pathways, Networks, and Systems, Crete, October, 2004.
107. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," Conference on Complex Systems, Northwestern University, Evanston, October, 2004.
108. B.O. Palsson, "Genome-scale Assessment of Phenotypic Changes During Adaptive Evolution," Keynote Address, Heidelberg, October, 2004.
109. B.O. Palsson, "*Systems Biology: Bringing Genomes to Life*," Neural Information Processing Conference 2004, Vancouver, Canada, December, 2004.
110. B.O. Palsson, "Educational and research challenges in systems biology," CSUPERB Symposium, Los Angeles, January, 2005.
111. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," Keystone Symposium, Santa Fe, February, 2005.
112. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," Stanford University BIO-X Lecture, March, 2005.
113. B.O. Palsson, "Bringing Genomes to Life: The Use of Genome-Scale *in silico* Models," UC Berkeley, March, 2005.
114. B.O. Palsson, "New 'Dimensions' in Genome Annotation," Keystone Symposium, Denver, CO, April, 2005.
115. B.O. Palsson, "New 'Dimensions' in Genome Annotation," Institute for Systems Biology 4th Annual International Symposium, Seattle WA, April, 2005.
116. B.O. Palsson, "New 'Dimensions' in Genome Annotation," Keystone Symposium, Denver, CO, April, 2005.
117. B.O. Palsson, "New 'Dimensions' in Genome Annotation," Lewis Sigler Institute Seminar, Princeton University, April, 2005.
118. B.O. Palsson, "The plasticity of the *E. coli* metabolome during adaptive evolution," Metabolomics 2005, Tokyo, June, 2005.
119. B.O. Palsson, "New 'Dimensions' in Genome Annotation," SIAM Symposium, New Orleans, July, 2005.
120. B.O. Palsson, "New 'Dimensions' in Genome Annotation," BioScience 2005, Glasgow, July, 2005.
121. B.O. Palsson, "Systems Biology and Bioengineering Cirruculum at UCSD," FOSBE Conference, Santa Barbara, August, 2005.

122. B.O. Palsson, "New 'Dimensions' in Genome Annotation," 12th European Congress on Biotechnology, Copenhagen, August, 2005.
123. B.O. Palsson, "Genome Scale Analysis of *E. coli* Adaptation to New Growth Environments, Including Re-Sequencing and Expression Profiling," 13th Annual International Conference on Microbial Genomes, Madison, Wisconsin, September, 2005.
124. B.O. Palsson, "New 'Dimensions' in Genome Annotation," CCBR/MaRS Research Symposium, Toronto, September, 2005.
125. B.O. Palsson, "New 'Dimensions' in Genome Annotation," University of Illinois special seminar, Urbana-Champaign, October, 2005.
126. B.O. Palsson, "The construction of an integrated metabolic and transcriptional regulatory network," ICSB Yeast Systems Biology Workshop, October, 2005.
127. B.O. Palsson, "New 'Dimensions' in Genome Annotation," YSBN Workshop, Boston, October, 2005
128. B.O. Palsson, "New 'Dimensions' in Genome Annotation," Special Seminar at EPFL, Switzerland, November, 2005
129. B.O. Palsson, "Prospect for Cell-Scale *in silico* Models of Production Cell Lines, "Cell Line Development and Engineering Conference, St. Louis, November 2005.
130. B.O. Palsson, "New 'Dimensions' in Genome Annotation, Pacifichem Conference, Honolulu, December, 2005
131. B.O. Palsson, "New 'Dimension' in Genome Annotation," O.A. Hougen Symposium, Madsion, Wisconsin, February, 2006.
132. B.O. Palsson, "Genome-Scale Analysis of *E. coli* Adaptation to New Growth Environments, Including Re-Sequencing, Location Analysis and Expression Profiling," Systems Biology: Integrating Biology, Technology and Computation, Taos, New Mexico, March, 2006.
133. B.O. Palsson, "Systems Biology," University of Iceland, Reykjavik, March, 2006.
134. B.O. Palsson, "New 'Dimenions' in Genome Annotation," UCSF, May, 2006.
135. B.O. Palsson, "Uses of the NimbleGen oligo-arrays for re-sequencing bacterial genomes and for mapping their genome-scale transcriptional regulatory networks," University of Iceland, Reykjavik, May, 2006.
136. B.O. Palsson, "Metabolic Characterization of Bacterial Adaptation," The Second Scientific Meeting of the Metabolomics Society, Boston, June, 2006.
137. B.O. Palsson, "Reconstruction of the global human metabolic networks based on sequence annotation and legacy data," Amylin Pharmaceuticals Research Seminar, La Jolla, July, 2006.
138. B.O. Palsson, "Reconstruction of the genome-scale human metabolic network based on build-35 and bibliomic data," 2006 Synthetic Biology Symposium, Irvine, September, 2006.
139. B.O. Palsson, "," Geobacter Conference, University of Massachusetts, October, 2006.
140. B.O. Palsson, "Bottom-up reconstruction of the human metabolic network based on build-35 and bibliomic data," ICSB, Yokohama, October, 2006
141. B.O. Palsson, "The genetic basis for adaptation of *E. coli* to new growth environments," **Plenary Lecture**, IECA Conference, Jeju, Korea, October, 2006.
142. B.O. Palsson, "Systems biology and the reconstruction of the genome-scale human metabolic network," ACDP 2006 Annual Meeting, Costa Rica, November, 2006.
143. B.O. Palsson, "The genetic basis for adaptation," **Special Lecture**, Center for Plant Cell Biology, UCR, February, 2007.
144. B.O. Palsson, "The challenge of incorporating regulatory effect in genome scale networks," Metabolic Engineering Workshop, Bethesda, February, 2007.
145. B.O. Palsson, "New 'dimensions' in genome annotation," Bioinformatics: Building Bridges Symposium, University of Minnesota, April, 2007.
146. B.O. Palsson, "Genome-scale reconstruction of the human metabolic network: basic concepts and practical uses," Nutrigenomics and Beyond: Informing the Future, "National Academy of Sciences, Washington, DC, May, 2007.
147. B.O. Palsson, "Metabolic Modeling," Summit on Systems Biology, Virginia Commonwealth University, June, 2007.
148. B.O. Palsson, "Systems Biology and Metabolomics: Mapping the Human Metabolome," Metabolomics Society 3rd Annual Conference, Manchester, UK, June, 2007.
149. B.O. Palsson, "Reconstruction of the genome-scale transcriptional regulatory network in *E. coli*," First Q-bio Conference on Cellular Information Processing, Santa FE, August, 2007.

150. B.O. Palsson, "Bringing Microbial Genomes to Life: the use of genome-scale *in silico* models for discovery and development, BioKorea 2007, Seoul, September, 2007.
151. B.O. Palsson, "Systems Biology of SNPs," 7th International Workshop on Pharmacodynamics of AntiCancer Agents, Costa Rica, September, 2007.
152. B.O. Palsson, "Genome-scale human metabolic reconstruction: current and future applications, BMES 2007, Hollywood, September, 2007.
153. B.O. Palsson, "Reconstruction of the genome-scale transcriptional regulatory network in *E. coli*," Seminar at the University of Washington, October, 2007.
154. B.O. Palsson, "New Dimensions in Genome Annotation," The Richard S.H. Mah Lecture, Northwestern University, Evanston, October, 2007.
155. B.O. Palsson, "The Reconstruction of the Genome-scale Transcriptional Regulatory Network in *E. coli*., IGSB Seminar, University of Chicago, May 2008.
156. B.O. Palsson, "Reconstruction of the Genome-scale Metabolic and Transcriptional Regulatory Networks in *E. coli*, ASM General Meeting, Boston, June, 2008.
157. B.O. Palsson, "The Genetic basis for Adaptive Evolution in *E. coli*," 16th International Microbial Genomes Conference, Lake Arrowhead, September, 2008.
158. B.O. Palsson, "The Use of Genome Scale Models for Metabolic Engineering," **Plenary Lecture**, Metabolic Engineering VII, Puerto Vallarta, September, 2008.
159. B.O. Palsson, "Global Reconstruction of the Human Metabolic Network based on Genomic and Bibliomic Data," **Keynote Address**, IBC Discovery 2 Diagnostics Conference, San Diego, October, 2008.
160. B.O. Palsson, "Reconstruction of the Genome-Scale Human Metabolic map Based on Build-35 and Bibliomic Data," **The Ernst W. Bertner Memorial Award Lecture,** Systems Biology of Cancer, Houston, October, 2008.
161. B.O. Palsson, "Systems Biology: An Era of Reconstruction and Interrogation, AiChE Annual Meeting, Philadelphia, November, 2008.
162. B.O. Palsson, "Reconstruction of the genome-scale human metabolic network based on build-35 and bibliomic data," ICBE Conference on Biomolecular Engineering, Santa Barbara, CA January, 2009.

GRADUATE STUDENTS & POST DOCTORAL RESEARCHERS

LAST	FIRST	YEAR/ DEGREE	RESEARCH PROJECT	CURRENT POSITION
Applebee	Kenyon	Current		UCSD Doctoral Student
Allen	Timothy	2005/Ph D	Genome-scale integrated models	University of Virginia
Andreadis	Stylianos	1996/ Ph.D.	Dynamics of Retrovirus Mediated Gene Transfer	SUNY, Buffalo, New York
Asthagiri	Dilip	1994/ MS	Systemic Behavior of Growth Factor Networks	University of Delaware
Barret	Christian	Current/ POST		UCSD Post Doctoral Researcher
Becker	Scott	Current	Reconstruction and analysis of biochemical reaction networks	UCSD Ph.D. Student
Bell	Steve	2004- PGR		UCSD Post Doctoral Researcher
Cho	Byung-Kwan	Current/ POST		UCSD Post Doctoral Researcher
Charusanti	Pep	Current/ Post		UCSD Post Doctoral Researcher
Chuck	Alice	1995/ Ph.D.	Directed Retroviral Motion as a Means of Enhancing Gene Transfer for Gene Therapy	Amgen, Thousand Oaks, CA--Research Scientist
Covert	Markus	2003/ PhD	Metabolic Regulation in silico in E. coli	Post Doc, Cal Tech
Duarte	Natalie	2006/Ph D	Reconstruction and analysis of eukaryotic metabolic networks	Exponent

Edwards	Jeremy	1999/ Ph.D.	Fuctional Genomics and the Computational Analysis of Bacterial Metabolism	University of New Mexico
Elkins	James	Current/ POST		UCSD Post Doctoral Researcher
Fahland	Tom	1999-2000--PGR	Whole cell simulation of kinetics	Researcher-Genomatica
Famili	Iman	2003/ PhD	In silico Analysis of Metabolism In the single cellular Eukaryote, Saccharomyces cerevisiae	Researcher-Genomatica, Inc.
Feist	Adam	Current		UCSD Graduate Student
Fong	Stephen	2004/ PhD	Gene knockouts and adaptive evolution	Virginia Commonwealth University
Francis	Karl	1999/ Ph.D.	An Ex Vivo Study of the Cellular Fate Process of Migration in Immature Hematopoietic Cells	Researcher-Biocyte
Fu	Patrick	1999-2001-PGR	Expression analysis and growth experiments in yeast	University of Hawaii
Halberstadt	Craig	1991/ Ph.D.	Transtubular Bioreactor Design	Carolinas Medical Center, Carolinas Med. Center
Herring	Chris	2006 POST	Gene regulatory network in E. coli	Researcher, Mascoma
Herrgard	Markus	2004/ PhD	Regulatory network reconstruction and modeling	UCSD Post-Doc
Hua	Qiang	2004/ POST	Fluxomics analysis of microorganisms	UCSD Post-Doc
Javanmardian	Minoo	1991/ Ph.D.	High Density Photoautotrophic Algal Cultures: Photobioreactor Design and Cell Cycle Kinetics	Booz Allen Hamilton
Jamshidi	Neema	2002/M S-Bioinf.	Red Blood cell simulation	UCSD PhD Student
Joshi	Abhay	1988/ Ph.D.	Integrated Metabolic Dynamics in the Human Red Cell	Sr. VP, CTO, CoTherix
Joyce	Andrew	2007/Ph D		Philip Morris
Keasling	Jay	1991/ Ph.D.	Dynamics and Control of Bacterial Plasmid Replication	UC Berkeley, Berkeley, CA--Associate Professor
Kim	Byung-Soo	1995/ MS	Bone Marrow Cell Culture and Metabolism in Rotating Wall Vessels	Professor-Korea
Lee	Baekseok	2003-2005		Post Doctoral Researcher—Inha University
Lee	Gyun-Min	1990/ Ph.D.	Production of Monoclonal Antibodies Using Free and Immobilized Hybridoma Cells	Korean Advanced Institute of Science and Technology
Lee	Choul-Gyun	1994/ Ph.D.	Photobioreactor Engineering: High-Density Algal Cultures Using Light-Emitting Diodes	Inha University, Korea--Asst. Professor
Lee	I-Der	1992/ POST	Computer Model of Red Cell Metabolism	MERCK--Sr. Res. Scientist
Lewis	Nathan	Current		UCSD Doctoral Student
Levee	Minette	1993/ MS	Encapsulated Bone Marrow Cultures as a Potential Assay for Human Hematopoietic Progenitors	
Mandalam	Ramkumar	1994/ Ph.D.	Nutritional Effects in High Density Chlorella Vulgaris Cultures	Cellerant Therapeutics
Marciniak	Jennifer	2002/M S	Bacterial evolution	UCSD Ph.D. Student
Merritt	Stephen	1992/ MS	MRNA Levels for Heavy and Light Chains in Stressed Hybridoma Cells	
Mo	Monica	Current		UCSD PhD student
Oh	Duk Jae	2000/ POST	Development of Bioreactors for Bone Marrow Cell Culture	Korean Advanced Institute of Science and Technology
Ozturk	Asuman	1990/ MS	Drug Dissolution Across Ethylcellulose Matrices	CHIRON, Emoryville, CA--Research Scientist

Ozturk	Sadettin	1990/ Ph.D.	Kinetic Characterization of Hybridoma Growth and Monoclonal Antibody Production Rates	MedImmune, Inc
Paek	Se-Hwan	1992/ POST	Continuous Bone Marrow Perfusion Chamber	Korean Advanced Institute of Science and Technology
Papin	Jason	2004/ PhD	Signaling networks	Asst. Prof—University of Virginia
Price	Nathan	2005/ PhD	Single cell models	Post Doctoral Fellow—Institute for Systems Biology
Peng	Ching-An	1995/ Ph.D.	Hematopoeitic Tissue Engineering: Effects of Fluid Dynamics and Mass Transfer on Cell Growth	USC, Los Angeles, CA--Asst. Professor
Portnoy	Vasily	Current		UCSD Doctoral Student
Ramakrishna	Ramprasad	1999/ POST	Genomatics and Antibiotic Development	Physiome Sciences, New Haven, CT
Raghunathan	Anu	2002-2005	Adaptive evolution	Mt. Sinai Medical Center
Reed	Jennie	2006/Ph D	E. coli K12	Assistant Professor, UW, Madison
Resendis-Antonio	Osbaldo	2005-2007		UNAM
Savinell	Joanne	1991/ Ph.D.	A Quantitative Study of Hybridoma Metabolism and Monoclonal Antibody Production	Cleveland State University, Cleveland, OH--Asst. Professor
Schellenberger	Jan	Current		UCSD Doctoral Student
Schilling	Christophe	2000/ Ph.D.	On Systems Biology and the Pathway Analysis of Metabolic Networks	Genomatica, Inc. San Diego, CA
Schwartz	Richard	1991/ Ph.D.	Development and Design of Ex Vivo Bone Marrow Bioreactor Systems	Avrion
Shen	Bing-Qian	1993/ POST	Optimizing Retroviral Titers	Metabolex, San Francisco, Research Scientist
Wiback (Smith)	Sharon	2004/ PhD	Applying kinetic constraints and regulatory logic to metabolic networks	Genomatica, Inc
Trakakis	Ionnanis	1986/ MS	Metabolic Dynamics in Cardiac Muscle	
Theile	Ines	Current		UCSD Doctoral Student
Varma	Amit	1993/ Ph.D.	Flux Balance Analysis of Escherichia Coli Metabolism	PDL BioPharma
Vo	Thuy	2006/Ph D.	H. pylori model	Amgen
Yeung	Matt	2007/P OST		Researcher, Imperial College
Zengler	Karsten	Current		UCSD Post Doc
Zylla	Brian	1994/ MS	Shear Sensitivities of Human Bone Marrow Cultures	Upjohn, Kalamazoo, MI, Product Development Engineer

COURSES TAUGHT

COURSE	TOPIC	INSTITUTION
ChE 230	THERMODYNAMICS I.	Univ. Michigan
ChE 330	THERMODYNAMICS II.	Univ. Michigan
ChE 341	FLUID MECHANICS.	Univ. Michigan
ChE 342	HEAT AND MASS TRANSFER.	Univ. Michigan
ChE 417	BIOCHEMICAL TECHNOLOGY.	Univ. Michigan
ChE 460	CHEMICAL ENGINEERING LABORATORY II.	Univ. Michigan
ChE 466	PROCESS DYNAMICS AND CONTROL.	Univ. Michigan
ChE 516	DYNAMICS OF BIOLOGICAL SYSTEMS.	Univ. Michigan

ChE 542	GRADUATE TRANSPORT PHENOMENA.	Univ. Michigan
BioE 160A	METABOLIC ENGINEERING.	UCSD
BioE 160C	IN SILICO BIOOGY	UCSD
BioE 166A	TISSUE ENGINEERING	UCSD
BioE 241B	TISSUE ENGINEERING; METHODS..	UCSD
BioE 241C	TISSUE ENGINEERING; SPECIAL TOPICS.	UCSD
BioE122A	BIOSYSTEMS AND CONTROLS	UCSD
BioE 123	SYSTEMS BIOLOGY AND BIOENGINEERING	UCSD
BioE 203	BIOINFORMATICS III	UCSD
BioE 211	SYSTEMS BIOLOGY AND BIOENGINEERING I	UCSD
BioE 212	SYSTEMS BIOLOGY AND BIOENGINEERING II	UCSD
BioE 213	SYSTEMS BIOLOGY AND BIOENGINEERING III	UCSD

PATENTS

1. U.S. Patent No. 5,399,493, Methods and compositions for the optimization of human hematopoietic progenitor cell cultures
2. U.S. Patent No. 5,437,994, Method for the ex vivo replication of stem cells, for the optimization of hematopoietic progenitor cell cultures, and for increasing the metabolism, GM-CSF secretion and/or IL-6 secretion of human stromal cells
3. U.S. Patent No. 5,459,069, Device for maintaining and growing human stem and/or hematopoietics cells
4. U.S. Patent No. 5,534,423, Methods of increasing rates of infection by directing motion of vectors
5. U.S. Patent No. 5,605,822, Methods, compositions and devices for growing human hematopoietic cells
6. U.S. Patent No. 5,614,378, Photobioreactors and closed ecological life support systems and artificial lungs containing the same
7. U.S. Patent No. 5,616,487, Stabilized retrovirus compositions
8. U.S. Patent No. 5,635,386, Methods for regulating the specific lineages of cells produced in a human hematopoietic cell culture
9. U.S. Patent No. 5,646,043, Methods for the ex vivo replication of human stem cells and/or expansion of human progenitor cells
10. U.S. Patent No. 5,654,185, Methods, compositions and apparatus for cell transfection
11. U.S. Patent No. 5,670,147, Compositions containing cultured mitotic human stem cells
12. U.S. Patent No. 5,670,351, Methods and compositions for the ex vivo replication of human hematopoietic stem cells
13. U.S. Patent No. 5,672,494, Methods of increasing rates of infection by directing motion of vectors
14. U.S. Patent No. 5,688,687, Bioreactor for mammalian cell growth and maintenance
15. U.S. Patent No. 5,763,266, Methods, compositions and devices for maintaining and growing human stem and/or hematopoietics cells
16. U.S. Patent No. 5,804,431, Method, compositions and apparatus for cell transfection
17. U.S. Patent No. 5,811,274, Methods, compositions and apparatus for cell transfection
18. U.S. Patent No. 5,866,400, Methods of increasing rates of infection by directing motion of vectors
19. U.S. Patent No. 5,874,266, Targeted system for removing tumor cells from cell populations
20. U.S. Patent No. 5,888,807, Devices for maintaining and growing human stem and/or hematopoietics cells
21. U.S. Patent No. 6,143,535, Targeted system for removing tumor cells from cell populations
22. U.S. Patent No. 6,326,198 B1, Methods and compositions for the ex vivo replication of stem cells, for the optimization of hematopoietic progenitor cell cultures, and for increasing the metabolism, GM-CSF secretion and/or IL-6 secretion of human stromal cells

23. U.S. Patent No. 6,514,722 B2, Method and apparatus for selectively targeting specific cells within a cell population
24. U.S. Patent No. 6,524,797 B1, Methods of identifying therapeutic compounds in a genetically defined setting
25. U.S. Patent No. 6,667,034, Methods for regulating the specific lineages of cells produced in a human hematopoietic cell culture, methods for assaying the effect of substances on lineage-specific cell production, and cell compositions produced by these cultures
26. U.S. Patent No. 6,753,161 Optoinjection methods
27. U.S. Patent No. 6,804,385 Method and device for selectively targeting cells within a three-dimensional specimen
28. U.S. Patent No. 7,127,379 B2, Method for the evolutionary design of biochemical reaction networks

TECHNOLOGY TRANSFER ACTIVITIES

1. AASTROM BIOSCIENCES	Founded 1988	www.aastrom.com
2. ONCOSIS	Founded 1997	www.oncosis.com
3. ICELAND GENOMICS CORP.	Founded 1998	www.uvs.is
4. GENOMATICA	Founded 1999	www.genomatica.com
5. CYNTELLECT (Formerly Oncosis)	Founded 2001	www.cyntellect.com

Toward a Science of Metabolic Engineering

JAMES E. BAILEY

Application of recombinant DNA methods to restructure metabolic networks can improve production of metabolite and protein products by altering pathway distributions and rates. Recruitment of heterologous proteins enables extension of existing pathways to obtain new chemical products, alter posttranslational protein processing, and degrade recalcitrant wastes. Although some of the experimental and mathematical tools required for rational metabolic engineering are available, complex cellular responses to genetic perturbations can complicate predictive design.

THE METABOLIC ACTIVITIES OF LIVING CELLS ARE ACCOMplished by a regulated, highly coupled network of ~1000 enzyme-catalyzed reactions and selective membrane transport systems. However, metabolic networks that evolved in natural settings are not genetically optimized for the objectives important in practical applications. Hence, performance of bioprocesses can be enhanced by genetic modification of the cells.

Metabolic engineering is the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology. The opportunity to introduce heterologous genes and regulatory elements distinguishes metabolic engineering from traditional genetic approaches to improve the strain. This capability enables construction of metabolic configurations with novel and often beneficial characteristics. Cell function can also be modified through precisely targeted alterations in normal cellular activities. Examples in the manipulation of protein processing pathways, as well as of pathways involving smaller metabolites, will be highlighted here.

At present, metabolic engineering is more a collection of examples than a codified science. Results to date promise future technological benefits, as well as contributions to basic science, agriculture, and medicine. However, many studies have shown the feasibility of metabolic engineering methods without achieving the yields, rates, or titers (final concentrations) required for a practical process. Most experiments explore changes in a single gene, operon, or gene cluster. After a new strain has been created by such a manipulation, limitations arise that can in principle be addressed by subsequent genetic manipulation. An iterative cycle of a genetic change, an analysis of the consequences, and a design of a further change, analogous to that articulated for protein engineering (*1*), can be used to find an optimized strain. The few cases to date in which such a metabolic engineering cycle has been implemented have achieved success. An emerging base of strategies, tools, and experiences will aid in identifying, implementing, and refining which particular set of genetic manipulations is most effective in accomplishing a desired change in cellular function.

Recruiting Heterologous Activities for Strain Improvement

Cloning and expression of heterologous genes can serve several useful purposes, including extending an existing pathway to obtain a new product, creating arrays of enzymatic activities that synthesize a novel structure, shifting metabolite flow toward a desired product, and accelerating a rate-determining step. Introduction of a functional heterologous enzyme or transport system into an organism can result in the appearance of new compounds that may subsequently undergo further reactions. Difficulties in anticipating these further reactions are a central limitation of metabolic engineering.

Expression of a heterologous protein does not guarantee appearance of the desired activity. The protein must avoid proteolysis, fold properly, accomplish any necessary assembly and prosthetic group acquisition, be suitably localized, have access to all required substrates, and not encounter an inhibitory environment. Despite these potential barriers to the successful recruitment of heterologous cellular activities, the number and scope of positive experiments encourage further application of this approach.

Synthesis of new products is enabled by completion of partial pathways. The genetic and metabolic diversity that exists in nature provides a collection of organisms with a spectrum of substrate assimilation and product synthesis capabilities. However, many natural strains are imperfect from an applied perspective. Their performance can sometimes be enhanced by extension of their native pathways. Native metabolites can be converted to preferred end products by the genetic installation of a few well-chosen heterologous activities (Table 1).

For example, the final precursor in a current commercial process for ascorbic acid (vitamin C) synthesis is 2-keto-L-gulonic acid (2-KLG). One route to 2-KLG involves two successive fermentations. The first converts glucose to 2,5-diketo-D-gluconic acid (2,5-DKG) in *Erwinia herbicola*; the second fermentation, carried out in a species of *Corynebacterium*, transforms 2,5-DKG to 2-KLG. Researchers devised a way to convert glucose to 2-KLG in a single fermentation step by cloning the *Corynebacterium* enzyme 2,5-DKG reductase, which catalyzes the 2,5-DKG to 2-KLG conversion, into *E. herbicola* (*2*). A similar goal was achieved for 7-aminocephalosporanic acid (7ACA), the precursor for several semisynthetic cephem antibiotics (*3*).

Posttranslational modifications can influence the function of proteins. The types of modifications that occur can be affected by expression of cloned protein processing enzymes. For example, expression in Chinese hamster ovary (CHO) cells of β-galactoside α2,6-sialyltransferase (*4*) allows the formation of sialyl α2,6-galactosyl linkages on its surface glycoproteins. These terminal glycosylation linkages are normally absent from proteins produced in this industrial

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cell line, including cloned erythropoietin. Thus, this strategy should enable erythropoietin made in recombinant CHO cells to more closely resemble human erythropoietin, which is rich in these linkages (*5*). In another study, mouse cells displayed the human H blood group antigen after transfection with human DNA (*6*).

Transferring multistep pathways: Hybrid metabolic networks. The transfer of genes that encode entire biosynthetic pathways to a heterologous host can provide more industrially robust strains, enhance productivity, or permit the use of less costly raw materials. Moreover, such experiments are useful for exploring the regulation and function of a multistep metabolic pathway in a particular species.

Transferring entire antibiotic biosynthetic pathways to heterologous hosts has been facilitated by the clustering of the genes involved (*7*). Genes for the biosynthesis of actinorhodin were transferred from *Streptomyces coelicolor* to *Streptomyces lividans*, enabling the latter strain to produce actinorhodin (*8*). Subsequently, clustered erythromycin biosynthetic genes from *Streptomyces erythreus* were transferred to *S. lividans*, which then synthesized an antibiotic indistinguishable from erythromycin A (*9*). *Escherichia coli* carrying this cloned gene cluster did not synthesize the antibiotic, possibly because of low transcriptional activity of *Streptomyces* promoters in *E. coli*. The fungi *Neurospora crassa* and *Aspergillus niger*, which normally do not produce β-lactam antibiotics, synthesized penicillin V after transformation with a cosmid containing *Penicillium chrysogenum* DNA that encoded enzymes in the penicillin biosynthetic pathway (*10*).

Polyhydroxybutyrate (PHB), a storage product sequestered in large amounts by some bacteria under growth-limiting, carbon source–excess conditions, is a biodegradable polyester that already has small-scale applications. *Alcaligenes eutrophus* can produce not only PHB but, when supplied with different precursors, can synthesize various polyhydroxyalkanoate copolymers as well (*11*). Metabolic engineering of the synthesis of these and related polymers should provide greater control over the nature and quantity of the polymer produced and should also offer alternative production organisms. The PHB synthesis operon from *A. eutrophus*, which encodes PHB polymerase, thiolase, and reductase activities, has been used to transform *E. coli* (*12*). As in *A. eutrophus*, this recombinant *E. coli* accumulates PHB when the nitrogen source is depleted; PHB concentrations in these cultures reach 50% of the dry cell weight.

Assembly of pathways for simultaneous degradation of chloro- and methylaromatics by combining and refining of cloned pathway segments and regulatory systems from several different organisms exemplifies the iterative design of an effective hybrid organism (*13*). The ultimate strain thus far constructed contains five pathway segments obtained from three organisms. The biochemical and metabolic complexities of the degradation of mixed substrates and the resulting rationale behind each portion of this construction offer useful general perspectives on metabolic engineering strategies (*14*).

Creating new products and new reactants. Expression of biosynthetic genes for a secondary metabolite in a heterologous host that synthesizes its own different secondary metabolite can result in the construction of an array of enzymatic activities that yield novel products. Among the novel antibiotics that have been produced in recombinant strains of *Streptomyces* by such manipulations are mederrhodins A and B and dihydrogranatirhodin (*15*), 2-norerythromycins A, B, C, and D (*16*), and isovaleryl spiramycin (*17*).

Compounds new to the cell that result from a heterologous activity often undergo further reactions. In some cases, such as in the biosynthesis of indigo by *E. coli* that express *Pseudomonas putida* naphthalene dioxygenase (*18*), these subsequent reactions are essential components of the desired pathway. Another illustration of metabolic engineering to introduce a novel intermediate into a host involves recombinant *E. coli* that express the cloned tyrosinase gene from *Streptomyces antibioticus* (*19*). Synthesis by the recombinant *E. coli* strain of the pigment melanin, an ultraviolet light–absorbing compound with material and cosmetic applications, depends on a single critical catalytic step: the oxidation of tyrosine and L-dopa to dopaquinone by tyrosinase; the remaining reactions that yield melanin are apparently nonenzymatic. Melanin production is increased when another protein from *S. antibioticus* is coexpressed with tyrosinase. Although definitive evidence is not yet available, this second protein may provide a copper-donor function that activates apotyrosinase. Thus, increasing the expression of a cofactor-requiring protein as part of a metabolic engineering scheme may require the engineering of an increased supply of the cofactor as well.

Biodegradation of undesirable compounds can often be accomplished by host enzymes after a heterologous activity provides the initial attack on the target compound or compounds. For example, the expression of *Pseudomonas mendocina* toluene monooxygenase in *E. coli* enabled the efficient degradation of trichlorethylene, a suspected carcinogen and widespread pollutant (*20*). In *E. coli*, degradation can be induced by isopropyl-1-thio-β-D-galactoside or by a temperature shift, rather than by toluene, as occurs in *P. mendocina*. In addition, the engineered *E. coli* has degradation kinetics (no competitive inhibition, as with toluene) and cosubstrate

Table 1. Heterologous activities recruited to alter small metabolite and protein end products. The original metabolite serves as the substrate for the synthesis of the new product through a pathway involving the new intermediate. It is difficult to prove that the inserted activity alone is responsible for an altered phenotype. In each case discussed here, the observed change in cell function is consistent with the expected consequence of the newly installed gene or genes. *A. chrysogenum*, *Acremonium chrysogenum*; GDP, guanosine diphosphate, *F. solani*, *Fusarium solani*; *P. diminuta*, *Pseudomonas diminuta*.

Host organism	Original metabolite	Heterologous enzymes added (source organism)	New product (new intermediate)
E. herbicola (*2, 21, 22*)	2,5-DKG	2,5-DKG reductase (*Corynebacterium*)	2-KLG
A. chrysogenum (*3*)	Cephalosporin C	D-Amino acid oxidase (*F. solani*), cephalosporin acylase (*P. diminuta*)	7ACA [7-β-(5-carboxy-5-oxopentanamido)-cephalosporanic acid]
CHO cells (*4*)	Terminal β-galactosyl residues in *N*-acetyllactosamine sequences	β-Galactoside α2,6-sialyltransferase (rat)	Sialyl α2,6-galactosyl linkages
Mouse L cells (*6*)	Unsubstituted type II, *N*-acetyllactosamine glycoconjugate end groups	GDP-L-fucose: β-D-galactoside 2-α-L-fucosyltransferase (A431 human cell line)	H fucosyl α1→2 galactosy linkages

requirements (glucose instead of toluene) that are superior to those of the native host of this toluene monooxygenase activity. This example illustrates that transfer of a crucial enzyme activity to a different regulatory environment can render that activity useful for biotechnology.

New metabolites arising from the action of cloned heterologous enzymes may also undergo undesirable side reactions. The precursor of 7ACA in engineered *Acremonium chrysogenum*, produced as a consequence of the cloned D-amino acid oxidase, can also react with hydrogen peroxide to give a useless by-product, dramatically reducing 7ACA yield (*3*). Cloned degradation enzymes have led to metabolic dead ends in the sense that the host cannot convert their products further; in some cases these recalcitrant intermediates inactivate key catabolic enzymes (*14*). Other unexpected complications can arise when desired end products are similar to some native metabolite and are converted to another product by host enzymes. After observations of unexpectedly low yields of 2-KLG in a recombinant strain (*2*), it was found that 2-KLG was converted to L-idonic acid by endogenous 2-ketoaldonate reductase (2KR). Cloning, deletion mutagenesis, and homologous recombination of the mutated gene for 2KR into the chromosome were part of several steps undertaken to develop an engineered organism able to accumulate large amounts of 2-KLG (>120 g/liter) (*21, 22*). The present engineered metabolic pathway involving these constructs (Fig. 1) shows complex interactions of enzymes and substrates that were identified, characterized, and engineered in an iterative process.

Perfecting strains by altering nutrient uptake and metabolite flow. Increased growth rates, decreased nutrient demands for cell growth, and higher attainable cell densities have advantages in many different applications. The use of metabolic engineering to realize these objectives has been based on increasing the efficiency of nutrient assimilation, enhancing the efficiency of adenosine triphosphate (ATP) production, and reducing the production of inhibitory metabolic end products. In one of the earliest applications of recombinant DNA to the improvement of the metabolism of the commercial strain, the goal was improvement of the efficiency of carbon conversion into cell mass by *Methylophilus methylotrophus*, a strain developed as an animal feed material. The native route of nitrogen assimilation used by this bacterium is the glutamate synthase pathway, which consumes one ATP per nitrogen incorporated into glutamate. Nitrogen assimilation by means of glutamate dehydrogenase, a process absent from this organism, does not require ATP. In an effort to improve cell yield, glutamate dehydrogenase from *E. coli* was expressed in a glutamate synthase mutant of *M. methylotrophus* (*23*). The efficiency of carbon conversion was increased 4 to 7%.

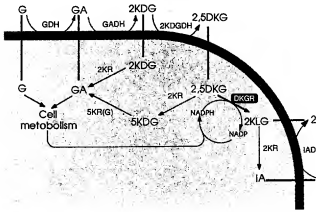

Fig. 1. Summary of the enzymes, intermediates, and by-products encountered in the synthesis of 2-keto-L-gulonic acid (2-KLG) from glucose in genetically engineered *E. herbicola*. The control of cloned (white printing on black) 2,5-DKG reductase activity, which requires the reduced form (NADPH) of nicotinamide adenine dinucleotide phosphate (NADP) supplied by the cell metabolism, directs metabolite flow to 2-KLG. Wide band with dots, cell membrane; straight bars, transport systems. Abbreviations are as follows: GDH, D-glucose dehydrogenase; GADH, D-gluconate dehydrogenase; 2KDGDH, 2-keto-D-gluconate dehydrogenase; DKGR, 2,5-diketo-D-gluconate reductase; IADH, L-idonate dehydrogase; 5KR(G), 5-keto-D-gluconate reductase (D-gluconate-producing); G, D-glucose; GA, D-gluconate; 2KDG, 2-keto-D-gluconate; 2,5KDG, 2,5-diketo-D-gluconate; IA, L-idonate; and 5KDG, 5-keto-D-gluconate. Reprinted by permission from (*21*).

End products of carbon catabolism (acetate, ethanol, and lactate) that inhibit cell growth are produced by bacteria, yeasts, and mammalian cells under conditions of oxygen limitation or carbon source excess. The final optical density of *E. coli* grown under shake-flask aerobic conditions was increased threefold after introduction of a plasmid that expressed pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* (*24*). The former activity, absent in unmodified *E. coli*, redirects catabolite fluxes from pyruvate and results in a shift from acetate production, which strongly inhibits cell growth, to production of ethanol, which is less inhibitory.

Microbial catabolic products such as ethanol, acetone, and butanol are important industrial chemicals. Large increases in ethanol yields from pentose and hexose sugar substrates from *E. coli* (*25, 26*) and *Erwinia chrysanthemi* (*27*) have been achieved by transformation with plasmids that encode pyruvate decarboxylase from *Z. mobilis*, in some cases coexpressed with *Z. mobilis* alcohol dehydrogenase. The *E. coli* so engineered have the potential practical advantages of rapid and efficient conversion of several sugars found in biomass (*26*).

α-Acetohydroxy acids, synthesized during fermentation by brewers' yeast, leak into the medium where spontaneous hydroxylation produces diacetyl, which has an undesirable flavor. On the basis of suggestions that the time required for beer lagering is determined by the time required for the enzymatic reduction of diacetyl by the yeast, genes for the enzyme α-acetolactate decarboxylase (α-ALDC) were cloned from *Klebsiella terrigena* or *Enterobacter aerogenes*, fused to yeast promoters, and inserted into *Saccharomyces cerevisiae* on multicopy plasmids. This enzyme converts α-acetolactate to acetoin, rather than diacetyl; acetoin influences flavor only at relatively high concentrations. Pilot brewing studies with these engineered strains that express α-ALDC yielded beer of quality equal to that produced by controls, but in a process time of 2 weeks, as compared to 5 weeks for the conventional process. The lagering step could be omitted when the recombinant brewers' yeasts were used because of low diacetyl production by these organisms (*28*).

Enabling a cell to utilize alternative materials as nourishment is another capability of metabolic engineering. In order to produce microbial surfactants from industrial waste raw materials, *E. coli* β-galactosidase and lactose permease were stably integrated into the chromosome of two *Pseudomonas aeroginosa* strains. These recombinant strains synthesize biosurfactants when grown in lactose and whey-based minimal media (*29*).

Yeast ornithine decarboxylase was cloned and expressed in cultured roots of *Nicotiana rustica* in order to direct a greater metabolite flux from ornithine to putrescine, a precursor of nicotine (*30*). Some clones showed approximately two times as much nicotine accumulation as the controls. Rearrangement of the native fluxes in the hyoscyamine-rich *Atropa belladonna* was motivated by greater commercial demand for scopolamine, the 6,7-epoxide of hyoscyamine. Expression of *Hyoscyamus niger* hyoscyamine 6β-hydroxylase in an *A. belladonna* hairy-root clone produced three to ten times as much scopolamine as did wild-type clones (*31*).

Systematic genetic manipulation of protein processing pathways has proven effective in increasing the quantity of active protein recovered. Overexpression of the *E. coli* chaperone proteins GroES and GroEL provided a five- to tenfold increase in assembled cyanobacterial Rubisco (D-ribulose-1,5-bisphosphate carboxylase/oxygenase) enzyme coexpressed in *E. coli*. In vitro studies of interactions among Rubisco and the GroE proteins implicate Mg^{2+}-ATP as a requirement for assembly (*32*). The failure to achieve assembly of Rubisco from higher plants in altered *E. coli* signals future challenges in the transfer of heterologous protein processing pathways (*33*). Other challenges include genetic manipulations of processing pathways of bacteria that alter the solubility of recombinant proteins (*34*). In addition, opportunities exist for extending such strategies to eukaryotic hosts (*35*).

Transfer of promising natural motifs: Vitreoscilla *hemoglobin*. Because of the constant drive toward maximum cell densities to maximize volumetric productivity, growth and product synthesis in many industrial processes are limited by oxygen supply. The Gram-negative aerobic bacterium *Vitreoscilla*, which lives in poorly aerated environments, synthesizes increased quantities of a hemoglobin molecule in oxygen-limited cultures (*36*). Although the function of this protein in its natural host has not been established, this pattern of regulation of expression, combined with the oxygen-binding and release characteristics of the protein, suggest a possible beneficial physiological activity in poorly oxygenated environments.

Motivated by this hypothesis and the premise that this beneficial function might be genetically transferred to industrial microorganisms, the gene for *Vitreoscilla* hemoglobin (VHb) was cloned and expressed in *E. coli* (*37*). *Escherichia coli* that carried a single copy of this gene integrated in the chromosome synthesized total cell protein more rapidly than an isogenic wild-type strain in oxygen-limited cultivations (Fig. 2), a response attributed to an increased efficiency of net ATP synthesis in the hemoglobin-expressing strain (*38*). Facilitation of oxygen transfer to the respiratory center (*39*) and modification of some aspect of cellular redox chemistry (*38*) have been suggested as contributing mechanisms for these phenomena. Coexpression of VHb increases the expression of cloned β-galactosidase, chloramphenicol acetyltransferase (CAT) (*38*), and α-amylase (*40*) by 1.5- to 3.3-fold relative to controls in oxygen-limited *E. coli* cultures, probably as a result of enhanced net ATP synthesis.

Aeration of bioreactors used in the synthesis of antibiotics is frequently complicated by the thick broths that result from growth of filamentous fungi and *Streptomyces*. Success of the strategy for enhancing aerobic metabolism in other bacteria prompted cloning and intracellular expression of VHb in two different *Streptomyces* species (*41*). *Streptomyces lividans* with a multicopy hemoglobin expression plasmid achieved final cell densities up to 54% greater than the untransformed host in shake-flask cultivations. The presence of cloned intracellular VHb in *S. coelicolor* markedly increased secondary metabolite accumulation, without affecting cell growth relative to a control strain containing a mutated VHb gene (Fig. 2).

These examples suggest a general genetic strategy for addressing stresses and corresponding productivity limitations encountered in bioprocessing: after identifying a response in nature to a similar stress (most likely involving a different organism), genes that specify that response can be transferred to the organism of choice.

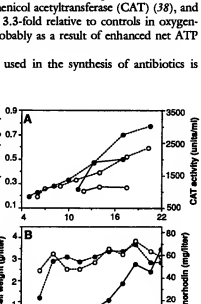

Fig. 2. (**A**) Time trajectories of total *E. coli* cell protein (dashed lines) and cloned CAT activity (solid lines) in the wild type (open symbols) and an engineered host that expresses VHb from a single gene copy integrated in the chromosome (closed symbols) in oxygen-limited fed-batch fermentations [reprinted from (*38*)]. (**B**) Cell densities (dashed lines) and concentration of actinorhodin in the medium (solid lines) in batch fermentations of *S. coelicolor*. Closed symbols are from a transformant expressing active VHb; open symbols are from a control transformant not expressing VHb [reprinted from (*41*)].

Redirecting Metabolite Flow

Typically the route of reactions to a desired product passes several forks where intermediates can enter alternative pathways. At such bifurcations of metabolite flow, a common resource—for example, substrate, enzyme, transport system, or ribosome—contributes to two or more parallel processes. Maximizing product formation requires that the desired route at each fork be made a priority and that traffic in alternative pathways be minimized to the extent possible without decreasing cell viability.

Directing traffic toward the desired branch. Amplification of the activity initiating a desired process at a fork in a metabolic flow is a common strategy of metabolic engineering. Whereas isolation of mutant enzymes that are desensitized to feedback repression was achieved with classical methods, such mutants may now be obtained more rapidly with the use of cloned genes. This approach also avoids the complication of uncharacterized additional mutations that are often obtained with classical, whole-cell mutagenesis.

The past decade has seen a new generation of strain improvements in amino acid–producing coryneform bacteria with metabolic engineering (also called molecular breeding) (*42, 43*). Central to the success achieved was the development of new vectors and transformation procedures.

Genetic engineering of improved threonine production by *Brevibacterium lactofermentum* illustrates some of the strategies useful in redirecting metabolite flow to the desired product. Figure 3 presents an abbreviated diagram of the reactions involved in the synthesis of the aspartate family of amino acids and a few key reactions that feed into the synthesis pathway for this family. Homoserine dehydrogenase (HD) was amplified by cloning and transformation into a threonine- and lysine-producing mutant (designated M-15). This mutant organism was selected for its lack of feedback inhibition of aspartokinase by threonine and lysine and of HD by threonine (*44*). The respective final concentrations of threonine, homoserine, and lysine from benchtop fermentations were 25.0, 2.8, and 1.1 g/liter for the recombinant strain compared to 17.5, 0.5, and 12.1 for M-15. Subsequent further engineering to coexpress cloned homoserine kinase (HK) with HD further increased the final threonine concentration to 33 g/liter and reduced homoserine and lysine levels, relative to the strain with cloned HD alone (*45*). In another study, the coryneform gene for HD was mutagenized to eliminate feedback inhibition by threonine. Introduction of this mutated HD gene into a lysine producer shifted the final lysine concentration from 65 g/liter to 4 g/liter and the final threonine concentration from 0 g/liter to 52 g/liter (*43*). Threonine production by M-15 was increased 12% by the expression of cloned phosphoenolpyruvate (PEP) carboxylase (PEPCase) (*46*). This manipulation was motivated by

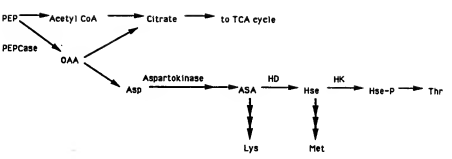

Fig. 3. Pathways of the biosynthesis of the aspartate family of amino acids. Metabolite abbreviations are as follows: acetyl CoA, acetyl coenzyme A; TCA, tricarboxylic acid cycle; Asp, aspartate; ASA, aspartate semialdehyde; Hse, homoserine; Lys, lysine; Met, methionine; Hse-P, O-phosphohomoserine; and Thr, threonine.

the researchers' desire to increase oxaloacetate (OAA) production and thereby to increase carbon flow into amino acid production. Further improvements in rates of amino acid synthesis and yields will depend on a better understanding of mechanisms of regulation of gene expression and metabolite flow in these bacteria (*47, 48*).

Because of metabolic engineering, *E. coli* has become an industrially important producer of amino acids. Transformation by multicopy plasmids that contain tryptophan (*49*) and threonine (*50*) biosynthetic genes have increased production of these amino acids. A project to engineer phenylalanine production in *E. coli* showed that overexpression of some genes in the phenylalanine biosynthetic pathway could cause a decrease in phenylalanine production and that inducible excision vector technology can be used to manipulate the biosynthesis of tyrosine, an inhibitor of the desired pathway (*51*). An intermediate in a metabolic pathway can be overproduced by combining a mutation that blocks that intermediate's use by the cell and by genetic augmentation of precursor flow into that pathway; although this concept has been used extensively in classical genetic production of organisms that are amino acid overproducers, it can be implemented in other contexts by metabolic engineering (*52*).

The gene *eryF* in *Saccharopolyspora erythrae* encodes the first enzyme in the pathway from 6-deoxyerythronolide B to the antibiotic erythromycin. After the targeted disruption of this gene using an integrative plasmid, 6-deoxyerythronolide B was converted to an erythromycin derivative that is more stable at the low pH of the stomach (*53*).

Because enzyme activities involved in secondary metabolite production are regulated at both the gene and protein levels, identifying genetic changes that accelerate synthesis of these metabolites is challenging. One successful strategy is based on measuring the biosynthetic pathway intermediate concentrations in the growth medium. Relatively high extracellular concentrations of the intermediate penicillin N suggested that the activity that converts this intermediate to cephalosporin C (encoded in *cef* EF) may limit the rate of the overall pathway (*54*). Thus, expression of *cef*EF was elevated through increased gene dosage in a production strain of *Cephalosporium acremonium*. This recombinant fungus exhibited a 15-fold reduction in penicillin N production and an increase of ~15% in cephalosporin C production.

Routing through protein processing pathways has also been altered by manipulation of host genes. The overproduction lethality commonly observed with exported β-galactosidase fusion proteins in *E. coli* is suppressed by the overproduction of *E. coli prl*F (*55*), and the expression of *E. coli* DnaK enables export of *lacZ*-hybrid proteins that are otherwise confined to the cytoplasm (*56*). An NH_2-terminal methionine often differentiates cloned polypeptides synthesized in *E. coli* from their native human counterparts. Coexpression of cloned *E. coli* methionine aminopeptidase with human interleukin-2 in *E. coli* has substantially reduced the fraction of product with methionine at its NH_2-terminus (*57*). Observation of large quantities of a variant of human tissue plasminogen activator (tPA) associated with GRP78 in the rough endoplasmic reticulum of CHO cells suggested that GRP78 binding was a rate-limiting step in tPA secretion. (GRP78 is the 78-kD glucose-regulated protein, one of the stress-response proteins.) Coexpression of antisense GRP78 message resulted in smaller quantities of GRP78 and faster tPA secretion (*58*).

A quantitative study was conducted of *S. cerevisiae* isolates that contain different numbers of the phosphoglycerate kinase (PGK) gene (*PGK1*). In some cases a 10 to 15% increase in PGK activity gave rise to a higher (30%) overall cell mass yield when the yeast were grown on glucose. However, in another construct that contained more copies of *PGK1*, yield was depressed by 40% (*59*). These results show the importance of fine-tuning the amount of gene or enzyme amplification to achieve the desired benefit.

Reducing competition for a limiting resource. Computer simulations with a detailed single-cell model suggested that competition between vector- and host-encoded messages for a common pool of ribosomes could limit cloned gene expression (*60*), a prediction consistent with experimental observations of reduced ribosome content in recombinant *E. coli* that contain more plasmids per cell (*61, 62*). By expressing a cloned mutant 16S ribosomal RNA, a population of ribosomes is created that is specialized for expression of only those cloned gene transcripts that bear a corresponding mutation in the Shine-Dalgarno sequence (*63*). Large amounts of messenger RNA transcribed from the cloned gene will not interact with the primary population of native ribosomes; therefore the expression of cloned genes will not interfere with the simultaneous expression of host cell genes important for protein synthesis. With this approach, expression of cloned β-galactosidase was increased by 35%. A 30% reduction in specific growth rate occurred after expression of the specialized 16*S* RNA; however, no growth rate reduction was observed on induction of cloned β-galactosidase synthesis (*64*).

Revising metabolic regulation. Positive control genes have been found so far in the biosynthetic gene clusters for actinorhodin, bialaphos, streptomycin, and undecylprodigiosin, which are all secondary metabolites produced by *Streptomyces* species. Cloning additional copies of an activator gene in the wild-type host can substantially increase antibiotic production, as indicated for undecylprodigiosin (*8*).

Modification of the regulation of expression of maltose permease and maltase is the basis for a metabolically engineered baker's yeast intended to reduce the time for leavening of sweet doughs (*65*). Glucose normally represses expression of these proteins and thereby blocks simultaneous maltose utilization. The engineered strain uses constitutive yeast promoters for these enzymes to enable simultaneous uptake and catabolism of both sugars. This is one of few examples in which a transport system has been manipulated.

A genetic engineering strategy to stimulate CO_2 production by bakers' yeast seeks to consume ATP (*66*). This could relieve ATP inhibition of phosphofructokinase (PFK) and pyruvate kinase, two regulatory enzymes in sugar catabolism. A futile cycle with PFK

was created by expressing cloned yeast fructose 1,6-bisphophatase (FBPase) from a yeast glycerophosphate dehydrogenase promoter that is induced by glucose; FBPase is not normally expressed in the presence of high glucose concentrations. This yeast strain produced 20 to 25% more CO_2 than the wild type.

In order to construct a pathway for processing the pollutant 4-ethylbenzoate, it was necessary to alter the regulation of the alkylbenzoate degradation pathway encoded on the *Pseudomonas* TOL plasmid (67). Originally 4-ethylbenzoate did not induce transcription of the crucial *meta* operon. Mutations were introduced into the positive regulator of *Pm* (the promoter of the *meta* operon) that enabled *Pm* activation by 4-ethylbenzoate.

Completing the Metabolic Engineering Cycle: Potentials and Perils of Rational Design

The iterative cycle of genetic modification, analysis of the metabolic consequences of this change, and choice of the next genetic modification has been successfully implemented in a few instances with promising results. Contemporary concepts and technologies for each function in this cycle are summarized next.

Cloning in industrial strains. The lack of suitable vectors and methods for the introduction of exogenous DNA limits the application of metabolic engineering in many important industrial organisms (68). Electroporation and conjugation have proven useful in introducing DNA into diverse organisms. The stable propagation of cloned genes remains problematic even in such a well-studied system as *Bacillus subtilis* and is apparently a result of the error-prone rolling-circle replication mechanism used by many plasmids in Gram-positive bacteria. Extensive rearrangements and deletions of both chromosomal and plasmid DNA occur frequently in some species, complicating their systematic manipulation. Restriction (cleavage) of heterologous DNA is a limitation in efficient engineering of many cells of practical interest.

The hurdles to be surmounted in developing the necessary genetic tools are illustrated in research that is establishing a foundation for engineering the complex catabolic metabolism of *Clostridium acetobutylicum*. This bacterium is the basis for the biological production of the industrial chemicals acetone and butanol. Efficient transformation of this organism required optimization of an electroporation protocol, and it was discovered that, because of a clostridial restriction enzyme system, *E. coli* is not a suitable organism for the cloning of clostridial DNAs, whereas *B. subtilis* is (69). Technology for chromosomal integration should soon follow, as several *C. acetobutylicum* genes have now been cloned.

Dissecting physiological responses. For the most effective design of a subsequent genetic manipulation, it is useful to know the concentrations of intracellular proteins and metabolites. The concentrations of many cellular proteins can be determined in principle from two-dimensional gel electrophoresis, but data bases are necessary to identify individual proteins (70). In vitro assays of changes in activities of key enzymes have been widely applied.

A broad spectrum of analytical methods can be applied for determining metabolite concentrations. The measurement of concentrations and in some cases of fluxes in particular pathways of interest can often be aided by the application of isotopically labeled precursors. For example, with the use of labeled acetate and glutamate, along with quasi-steady state conservation equations for intracellular metabolites, the velocities for carbon flow through *E. coli* growing on acetate can be determined (71).

Nuclear magnetic resonance (NMR) spectroscopy has been applied to estimate metabolite concentrations in whole cells, cell extracts, and growth media (72). For example, ^{31}P NMR measurements of *S. cerevisiae* cells converting glucose to end products under anaerobic conditions, in concert with a methodology for extracting individual component information from the sugar phosphate portion of the spectrum, provided estimates of metabolite concentrations that were essential for analysis of the pathway (73). The time and instrumentation required to evaluate metabolite concentrations presently limits rational metabolic engineering.

Design principles and cell models: Coping with complexity and coupling. No universal principles have emerged from metabolic engineering research to guide the choice of the next useful genetic alteration. Attempts to address these problems with artificial intelligence have shown that there is no substitute for knowledge of the pathways involved, their regulation, and their kinetics. Some useful approaches include measurements of intermediate concentrations to indicate possible rate-determining reactions, genetic transfer of natural stress response motifs, and applications of organisms that can be used over wide ranges of temperature and pH (74).

Alternatively, if a mathematical description of the system is available, sensitivity analysis can be applied to calculate the expected response of the pathway to changes in the individual steps or pathway segments. An advantage of such an approach is its simultaneous determination of the sensitivities of the desired flux to many different participating reactions, permitting the identification of situations in which several genetic modifications in concert are required to achieve a desired response.

A body of theoretical developments known as metabolic control theory is well suited to the requirements of rational metabolic engineering (75). A central result provided by this theory is a sensitivity calculation that provides the flux control coefficients, defined as the fractional changes of flux expected for a unit fractional change in the amount of each enzyme participating in a given pathway. In addition, it is possible to evaluate the sensitivity of flux through the pathway to individual parameters in kinetic expressions for each of the enzymes, thereby providing guidance for useful protein engineering to accelerate the pathway. Analysis of several simple examples that involve unbranched sequences of reactions showed that sole control of flux by any single step (in other words the existence of a single, rate-limiting step) is in general not expected. Instead, the flux through the pathway is usually influenced by the activities of several individual steps. This result, augmented by specific model calculations outlined below, provides motivation for sequential improvement of metabolic pathways.

In one of the few cases in which detailed kinetic expressions for each step in the reaction network, as well as the concentrations of all substrates and effectors, are known or estimated, flux control coefficients were determined for nongrowing yeast converting glucose to ethanol and other end products (76). Several general points are suggested by this investigation. First, the sensitivity of pathway flux to individual step changes depends on the environment in which the cell is grown. It is therefore important to carry out modeling and measurement under the expected industrial conditions. Second, flux control can be extraordinarily sensitive to some parameters such as intracellular pH. Third, in any system with interacting pathways (and it is difficult to envision any case where this does not occur), the most general version of metabolic control theory must be used (77). Such coupling is extensive in the usual case of growing cells, where the pathway of interest interacts with all of the other metabolic processes in the cell. A strategy for accomplishing flux control coefficient calculations in this situation has been presented (78). Finally, calculations with the kinetic model formulated in a yeast biocatalysis study indicate that amplification of the activity of one enzyme results in a shift of flux control to other steps in the pathway. New theory that presumes linear approximations for all rate expressions provides estimates of flux control coefficients,

without requiring knowledge of kinetics and using time-resolved metabolite concentration measurements instead (*79*); the practical merits of this approach have not yet been evaluated.

Both sensitivity to small changes and simulations of responses to large changes in intracellular activities can be calculated from a detailed and reliable mathematical model of the cell. Large quantities of biological information have been integrated into computer models for single cells (*60, 80*) and several molecular control systems (*81*). These have successfully simulated consequences of several genetic and environmental changes. Although useful initial directions for genetic improvement have been suggested by such models, they have not yet been used as the central tool in an iterative metabolic engineering study. In spite of their obvious limitations, these mathematical structures are the only way that the net consequences of simultaneous, coupled, and often counteracting processes can be simulated and evaluated consistently and quantitatively.

Minimizing response cascades. Unanticipated cell responses to a genetic modification may complicate rational practice of the metabolic engineering cycle. Introduction of a cloning vector alone may result in a large cascade of metabolic changes, many of which are difficult to anticipate. For example, the introduction of multicopy plasmids into *E. coli*, even without overexpression of a cloned product, has been shown to cause substantial changes in growth rates, cell cycle regulation, amounts of many individual proteins, glucose uptake, and carbon catabolite production rates (*82*). Transformation of yeast with multicopy plasmids can introduce lesions that persist after the plasmids have been eliminated from the transformants (*83*). Different mammalian cell clones transfected by the same vector often exhibit different growth rates and cell sizes. Therefore, introduction of a desired genetic change should be carefully configured to minimize perturbation of the host, using the lowest gene dosage and lowest expression level that give the desired result. The apparatus used for selecting the modified strain should also be carefully considered. For example, ampicillin resistance used for the maintenance of many laboratory recombinant *E. coli* strains is provided by cloned β-lactamase. This precursor must be processed at the cytoplasmic membrane in competition with host cell preprotein, which often results in a major physiological disruption.

Even if the genetic manipulation is accomplished in a relatively well-controlled fashion, the regulatory apparatus of the cell at both the gene and protein levels may confound the intended change or even alter cellular activities. For example, amplification of citrate synthase in *E. coli* did not increase the flux through the citric acid cycle because of a compensating modulation of the activity of isocitrate dehydrogenase (*84*). Expression of even low concentrations of unnatural proteins can activate stress responses, influencing many cell functions (*85*). Anticipating and accounting for such regulatory responses to genetic intrusions are fundamental challenges for the future.

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Network Rigidity and Metabolic Engineering in Metabolite Overproduction

GREGORY STEPHANOPOULOS AND JOSEPH J. VALLINO*

In order to enhance the yield and productivity of metabolite production, researchers have focused almost exclusively on enzyme amplification or other modifications of the product pathway. However, overproduction of many metabolites requires significant redirection of flux distributions in the primary metabolism, which may not readily occur following product deregulation because metabolic pathways have evolved to exhibit control architectures that resist flux alterations at branch points. This problem can be addressed through the use of some general concepts of metabolic rigidity, which include a means for identifying and removing rigid branch points within an experimental framework.

ALL ORGANISMS USE PRIMARY METABOLIC PATHWAYS TO supply precursor metabolites and energy to anabolic pathways that synthesize cellular constituents that are necessary for growth and maintenance. In many industrial strains of microorganisms (as well as tissue and plant cultures), these anabolic pathways have been exploited for the overproduction of compounds (such as amino and nucleic acids, antibiotics, vitamins, enzymes, and proteins) that cannot be synthetically produced or for which it is not economical to do so. In general, a particular metabolite is overproduced by deregulating the pathway directly associated with the synthesis of that metabolite, or, more recently, by transforming a robust host organism (typically *Escherichia coli*) with the genes that encode for the synthesis of the desired product (*1*, *2*). This approach, however, does not necessarily result in high product yields (defined as the moles of product formed per mole of substrate consumed) since carbon flux distributions at key branch points (nodes) in the primary metabolism [such as glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway] must often be radically redirected from the flux distributions that are normally associated with balanced growth. Such metabolic flux alterations are often directly opposed by mechanisms for controlling enzyme activity that have evolved to maintain flux distributions that are optimal for growth. We refer to this inherent resistance to flux alterations as metabolic or network rigidity and to the genetic modifications of specific nodes in the primary metabolism for the purpose of enhancing yield and productivity as metabolic engineering (*3*). Although genetic manipulations can now be readily performed, there are relatively few accounts of successful metabolic flux alterations because of the complex, nonlinear nature of the metabolic control architectures.

The nature and types of metabolic rigidity are reviewed in this article along with methods to identify and possibly circumvent such undesirable nodal controls. The overproduction of lysine by *Corynebacterium glutamicum* [and related strains (*4*)] is used as a vehicle to illustrate key points because of: (i) the lack of compartmentalization in bacteria; (ii) the need for significant flux alterations to optimize lysine biosynthesis; and (iii) the apparent marginal success of mutation-selection (*5*, *6*) or genetic engineering (*7*) techniques used to that end. The concepts, however, are of general value, and the methods are applicable to other metabolic products as well.

Basis of Metabolic Rigidity

Although intracellular metabolite concentrations can fluctuate during growth, on average, the distributions of the major cellular groups (proteins, RNA, DNA, lipids, and so forth) remain relatively proportional to one another throughout balanced growth (*8*). In fact, metabolites and energy required to synthesize an *E. coli* cell have been calculated on the basis of its known composition (*9*). In order to preserve this regularity in cellular composition, the primary metabolism has evolved coordination of pathway control, such that building-block metabolites, energy [such as adenosine triphosphate (ATP)], and biosynthetic reducing power [such as nicotinamide adenine dinucleotide phosphate (NADPH)] are synthesized in approximate stoichiometric ratios during balanced growth. Al-

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IN MEMORIAM

Jay Bailey (1944–2001)

Jay Bailey passed away in Zurich on 9 May 2001 at the age of 57. Jay received his education at Rice University, graduating with a B.A. in 1966 and Ph.D. in 1969, both in chemical engineering. After a short period with Shell Development he joined the Chemical Engineering Faculties of the University of Houston in 1971 and Caltech in 1980. In 1992 Jay was appointed Professor of Biotechnology at the Swiss Federal Institute of Technology (ETH) in Zurich.

In his early years Jay studied extensively the dynamics of chemical reactions and reaction networks with particular emphasis on the origin and interpretation of autonomous and forced oscillations of chemically reacting systems. During the 1970s, his interest shifted gradually to biological systems and this shift was culminated with the publication in 1977 of the landmark textbook *Biochemical Engineering Fundamentals* (coauthored with D. F. Ollis). At the time of his passing, Jay had coauthored approximately 400 publications, mostly in the field of biotechnology, including many seminal papers and visionary commentaries in leading journals.

Besides his outstanding contributions to biotechnology, Jay was instrumental in defining and building the foundations of metabolic engineering. He is arguably the first engineer who, in the early 1980s, embraced and promoted genetic engineering as an enabling new technology for improving cellular biocatalysts for industrial processes. This naturally led to the need to study the behavior of bioreaction networks in their entirety, analyze metabolic flux and flux control, and rigorously describe the physiology of wild-type and recombinant microorganisms. Although some of these questions had been investigated before by biochemists and chemical engineers, their sum total emerged as a distinct new field, metabolic engineering. Jay played a key role in setting the foundations and expanding this field.

Until the last day before being admitted to the hospital, Jay was actively working on the program of the 4th Conference of Metabolic Engineering, which he would have chaired in October of 2002. His plans for the conference program as well as his more recent scientific writings reveal a broader vision for metabolic engineering: First, metabolic engineering is at the forefront of functional genomics due to its efforts to describe the cellular physiology of wild-type microorganisms and recombinants with well-defined genetic backgrounds. Second, metabolic engineering provides an ideal *integrating platform* of genomic and physiological information and data by taking a holistic view of metabolic networks and cellular physiology for the identification of targets for genetic manipulation. As such, it is contributing concepts and methods of importance to systems biology. Finally, Jay expanded metabolic engineering beyond the original context of industrial strain improvement to also include mammalian cells, tissues, and medical applications.

Jay leaves a very rich legacy to the biotechnology and biochemical engineering communities. He mentored a school of distinguished students with brilliant careers in industry and academia and, through his teaching and writings, ushered biochemical engineering into the modern era of cellular and molecular biotechnology. Most importantly, he upheld the highest standards in education and research. These contributions make Jay undoubtedly the most influential biochemical engineer of modern time.

Jay was internationally recognized for his work in diverse areas of chemical engineering and biotechnology. He won numerous honors and awards in his career. The most recent was the First Merck Award in Metabolic Engineering. His award acceptance speech summarized his vision for metabolic engineering and formed the basis of the first perspective article in this journal (*Metab. Eng.* **3**, 111–114, 2001). We are pleased to be able to reprint below the rendition of Bob Dylan's song *They Are A Changin'* by singer–poet Jay Bailey.

In Jay's honor, *Metabolic Engineering* is instituting a Young Investigator Best-Paper Award in Metabolic Engineering to be awarded every 2 years to the author(s) of an outstanding paper published in *Metabolic Engineering*. Details about the administration of the award will be provided in a future issue.

The (Metabolic Engineering) Times,
They Are A 'Changin' (Dylan/Bailey)

Come gather 'round people wherever you roam,
And admit that the waters around you have grown,
And accept it that soon you'll be drenched to the bone.
If your time to you is worth saving,
Then you'd better start swimming or you'll sink like a stone,
For the times, they are a changin'. (Bob Dylan)

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Come gather Metabolic Engineers 'cross the land
At ME III we'll take command
Of cells that are too slow to produce or grow.
If it's higher fluxes you're needin'
Then we'll shift the controls, and block bad outflows.
For the times, they are a changin'.

Do you need a new molecule or neutraceutical
The Metabolic Engineer has the answers for you.
We'll import new pathways, and shuffle them too.
Is your lead compound library fadin'?
We'll give new adducts to your old natural products
For the times, they are a changin'.

Rational or random, which way is best?
Solving the problem passes the test.
Complex responses confuse the quest.
More genetic and array technologies
Will give us insights to networks' delights.
For the times, they are a changin'.

Genomes are in hand, the sequences there,
An amazing resource that we all share.
Genes and controllers, bioinformatics tells us where.
But how is all of this workin'?
Let's decipher a yeast, understand that at least.
For the times, they are a changin'.

How is phenotype controlled by the genes?
Nobody knows, least of all the machines.
Medicine will thrive if we can discover the means,
To merge our knowledge and information
And find genes' intent and control by environment.
For the times, they are a changin'.

Metabolic Engineers have all the tools—
Biology, computing, and engineering rules,
Knowledge, experience, perspective on detail.
Let's help Metabolic Genomics to set sail.
Opportunity's here . . . but now it's time for a beer!
For the times, they are a changin'. (Jay Bailey)
October 2000

Videocassettes of Jay's Merck Award talk at the 3rd Metabolic Engineering Conference are available through the Engineering Foundation. Contact Ms. Barbara Hickernel at 212-591-7836 or engfnd@aol.com.

Gregory Stephanopoulos
Co-Editor, *Metabolic Engineering*

Engineering of Molecular and Cellular Biocatalysts: Selected Contributions by James E. Bailey

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Abstract: James (Jay) E. Bailey was a pioneer in biotechnology and biochemical engineering. During his 30 years in academia he made seminal contributions to many fields of chemical engineering science, including catalysis and reaction engineering, bioprocess engineering, mathematical modeling of cellular processes, recombinant DNA technology, enzyme engineering, and metabolic engineering. This article celebrates some of his contributions to the engineering of molecular and cellular biocatalysts, and identifies the influence he had on current and future research in biotechnology. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **79:** 490–495, 2002.

Keywords: James Bailey; contributions to biotechnology and biochemical engineering; biocatalysis; metabolic engineering

INTRODUCTION

James (Jay) E. Bailey, a pioneer in biotechnology and biochemical engineering, succumbed to metastatic cancer on May 9, 2001. After receiving his undergraduate and doctoral degrees at Rice University and a brief stint at Shell Development (Houston), he started his academic career in 1971 at the University of Houston. In 1980 he moved to the California Institute of Technology, and in 1992 he moved yet again to the Swiss Federal Institute of Technology (ETH, Zurich) where he was Professor of Biotechnology until his untimely death. During his 30 years in academia he made seminal contributions to many fields of chemical engineering science, including catalysis and reaction engineering, bioprocess engineering, mathematical modeling of cellular processes, recombinant DNA technology, enzyme engineering, and metabolic engineering. Here we summarize some of his contributions to the engineering of molecular and cellular biocatalysts.

Bailey's growing interest in biological systems in the 1970's and 1980's was no doubt due to his interest in chemical catalysis. So it was no surprise that he moved into the fields of enzyme technology and fermentation/cell culture. In essence, these areas are catalytic; whether it is in the use of an enzyme to catalyze biotransformations or a cell to produce specific compounds. Although he did not use enzymes synthetically, he did set the stage for research that would lead to several generations of enzymologists, chemists, and biochemical engineers who use enzymes in organic synthesis. By laying the quantitative groundwork for immobilized enzyme systems, and studying their structure and function in synthetically relevant forms, Bailey elevated biocatalysis from a synthetic oddity to a promising technology.

In the mid-1980's, shortly after Bailey started to acquaint himself with the principles and practice of emerging recombinant DNA technologies, he came to realize that the science of metabolism presented a particularly fertile ground for a chemical engineer to apply this new technology. To emphasize the marriage between metabolic biochemistry and chemical engineering science, he coined the term "metabolic engineering." By the mid-1980's he had set his sights squarely on the twin goals of developing the fundamentals of metabolic engineering and identifying interesting applications within this new area of applied science that would highlight its longterm potential to the broader scientific community. This was to remain a dominant theme in his research program for the remainder of his career. His vision also inspired an entire generation of students from his own and other laboratories, many of who continue to evolve the frontiers of metabolic engineering to this day.

IMMOBILIZED ENZYME TECHNOLOGY

When compared to their chemical counterparts, biocatalysts are exquisitely selective and highly reactive over a broad range of operating conditions. Moreover, whole microbial cells (primarily bacterial and fungal) and their catalytic machinery (e.g., enzymes and metabolic pathways) can accept a wide array of complex molecules as substrates, yielding products with unparalleled chiral (enantio-), positional (regio-), and chemoselectivities. Such high selectivity affords efficient reactions with few byproducts, and ensuring that

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enzymes can be used in both simple and complex transformations without the need for tedious blocking and deblocking steps that are commonplace in enantio- and regioselective organic synthesis.

Structure and Function of Enzymes on Solid Supports

Prior to Bailey's arrival in the enzyme technology arena, the vast majority of biocatalysts, even for commercial applications, involved soluble enzymes. Nonetheless, heterogeneous preparations offer a variety of advantages over homogeneous ones for scaled-up biocatalytic operation, including retention of the enzyme in the bioreactor, use of packed-bed operation, and high catalyst loading. Immobilized enzymes, however, also display different structural and functional properties, as well as being subject to substrate diffusional limitations that impact observed enzyme activity and stability.

As early as 1977, Bailey recognized that immobilization could result in operational advantages for biocatalytic systems. For example, glucose oxidase, an enzyme that is rapidly inactivated by H_2O_2 could be stabilized dramatically upon immobilization, even using simple covalent attachment techniques. Various supports were used, including activated carbon, glass, metal oxides, and polymer resins (Bailey and Cho, 1983; Cho and Bailey, 1978, 1979). These early studies were augmented by quantitative evaluation of enzyme systems on solid supports, with direct comparison to soluble systems. Bailey and others found that the observed reaction kinetics could be affected by the nature of the immobilization support. In one of the first applications of electron paramagnetic resonance (EPR) spectroscopy applied to immobilized enzymes, Bailey, along with then graduate student Douglas Clark, demonstrated that immobilization affects the structure and dynamics of the mammalian protease, α-chymotrypsin (Clark and Bailey, 1984). Structure-function studies were performed, which resulted in a firm understanding of the improvement in enzyme activity upon the use of linkers to attach enzymes to the support rather than direct enzyme attachment. Simultaneous to this study was the ability to quantify the influence of substrate diffusional limitations on heterogeneous enzymatic catalysis (Clark et al., 1985; Dennis et al., 1984). This study, expanded many fold by others on a wide range of commercially relevant enzymes, provided a quantitative foundation for immobilized enzyme technology that is critical today in the food, pharmaceutical, and chemical industries, and in both aqueous and nonaqueous media. Above all else, these studies led to the realization that enzymes [and whole cells (Doran and Bailey, 1986)] were capable of being manipulated simply by the environment that they were used in, and therefore, the enzyme technologist was not limited to the native properties of enzymes for eventual operation.

Nonaqueous Enzymology—Quantitative and Mechanistic Principles

Enzymatic catalysis in organic solvents has dramatically shaped the emerging use of enzymes in organic synthesis (Dordick, 1992; Klibanov, 1990). Nonaqueous media results in higher substrate solubility, reversal of hydrolytic reactions, modified enzyme specificity, and improved thermostability. Nonaqueous conditions enable one to tap into novel enzyme activities and selectivities heretofore only possible using genetic modifications or complex multistep pathways. Because such an approach is so attractive, enzymatic catalysis in nonaqueous media has undergone rapid expansion, particularly over the past decade. While there is little question that enzymes can function in nonaqueous media, reaction rates are typically quite low and enzymes often have limited stability in such environments. In nearly all cases, the catalytic activity displayed by enzymes in nearly anhydrous (or neat) organic solvents is far lower than in water; as much as five orders of magnitude lower! There is nothing unique about subtilisin', and similarly diminished activity of many enzymes (hydrolases and oxidases) is observed in organic solvents. Nevertheless, there may be nothing inevitable about this decline, and both its underlying causes and effective remedies are emerging.

While Jay Bailey did not study biocatalysis in organic media, nonetheless his prior research yielded the tools necessary to make critical contributions in this area, particularly for studying heterogeneous enzyme preparations quantitatively. For example, in the early 1990's, Affleck et al. (1992) and Xu et al. (1994), using enzyme kinetic, solution thermodynamic, and EPR spectroscopic techniques, uncovered an intriguing correlation between increased activity of enzymes in slightly hydrated organic solvents relative to dry solvents and the polarity of the enzyme's active site. Further quantitative studies involving pressure-based electrostriction resulted in one of the first transition-state mechanistic models of observed enzyme activity in nearly anhydrous environments. Specifically, Michels et al. (1998) used Kirkwood electrostriction analysis to show that the dipole moment of subtilisin's transition state was the same in hexane as in water. Hence, it is not surprising that the enzyme is much less active in the former than the latter, as water can stabilize charge separation in the transition state that gives rise to such a dipole moment. Prior to this result, Khmelnitsky et al. (1994) discovered that enzymes could be dramatically activated (nearly 4,000-fold) by lyophilizing them in the presence of a nonbuffer salt such as KCl. Based on the work by Michels et al. (1998), it was reasoned that charge stabilization could be achieved by nonbuffer salts, which provides a locally high polarity near the transition state of the enzyme in nonpolar organic media, even in the absence of added water. Indeed, the 1990's provided a wealth of techniques to activate enzymes for use in organic media. Some of the prominent ones include the addition of polyols (Adlercreutz, 1993), crown ethers (Engbersen et al., 1996), transition-state analogs (Slade and Vulfson, 1998), and substrates and substrate mimics (Braco et al., 1990; Rich and

Dordick, 1997). Beginning with a fundamental picture of enzymes in the nonaqueous milieu, the field of nonaqueous enzymology has recently seen cases where enzymes function in organic media as well as they do in water; again a striking realization that biocatalysis can be tailored by manipulating an enzyme's heterogeneous environment. Such a result can be traced back to Jay Bailey and his initial work with glucose oxidase and α-chymotrypsin on solid supports.

Activated biocatalyst formulations have begun to impact society in terms of human health (better drugs), the environment (more efficient and selective synthetic conditions), and industry (new routes to existing and novel chemicals and materials). For example, salt-activated thermolysin catalyzes the regioselective acylation of paclitaxel (taxol) in a synthetic scheme to produce a water-soluble prodrug (paclitaxel 2'-adipic acid), which may result in an easier mode of delivery of this anticancer compound (Khmelnitsky et al., 1997). The adipic acid derivative of taxol is $\approx$1700-fold more soluble in water than the native drug. In addition to the direct impact on taxol prodrug synthesis, activated biocatalysts are beginning to find applications in the synthesis of chiral molecules and in the resolution of optical isomers, both critical in the preparation of new pharmaceuticals.

Nonaqueous enzymology is quickly maturing, and combined with directed evolution (Affholter and Arnold, 1999) and gene-shuffling technologies (Powell et al., 2001), biocatalysts with tailored and controllable activities, stabilities, and selectivities will yield still better commercial catalysts. The ability to use enzymes in organic media has also led to the bridging of the materials and biological worlds, wherein enzymes and other proteins can be incorporated into polymers (organic and inorganic) to provide structure and function to the material (Wang et al., 1997). Opportunities in drug discovery, through protein chip technologies (Kim et al., 2001), and nanotechnology (Graff et al., 2001), through smart materials, will have a dominating impact in society for years to come. All of these advances can be linked to the fundamental immobilized enzyme studies initiated by Jay.

MANIPULATION OF CELLULAR FUNCTION

Three main reasons make metabolism a particularly interesting target for manipulation by a chemical engineer. First, in its most basic form, metabolism represents a large set (network) of chemical transformations operating in series/parallel in one pot. This has a direct analogy with, say, the chemistry in a naphtha cracker, the analysis of which is widely regarded as the fountainhead of modern chemical reaction engineering. Jay Bailey was only too aware of this, since his graduate training and early independent research focused on the theoretical and experimental analysis of complex reaction networks. Second, most practical limitations of cells as biocatalysts are due to metabolic constraints or defects. It therefore follows that the enhancement of existing biotechnological processes as well as the development of new ones rests heavily on a solid appreciation of the nuances of the underlying metabolism. Finally, the synthetic capabilities of metabolism (especially anabolic metabolism) are unparalleled. Thus, the ability to manipulate such powerful chemistry should be able to afford a virtually unlimited spectrum of new products. Since Bailey was especially interested in the practical applications of academic science, the latter two arguments also appealed to him.

What does a chemical engineer need to learn to tackle metabolic problems? Bailey thought long and hard about this right from the beginning. He was particularly aware that metabolic engineering would be unable to sustain itself as a burgeoning field if it simply represented a collection of interesting anecdotal examples involving the manipulation of cellular function. Indeed, the title of his landmark article in *Science* (1991) is testament to his constant desire for a strong foundation based on rigorously tested principles and cutting-edge tools. Examples of such principles and tools include:

1. *The chemical logic of metabolism.* While not apparent at first glance, the chemical logic of metabolism provides an excellent basis for organizing an overwhelmingly large body of information into a "sensible" network of chemical transformations. For example, an understanding of how and why energy is stored in the form of phosphoanhydride linkages in a cell allows one to appreciate the logic of many phosphorylation–dephosphorylation reactions that occur during central carbon metabolism (glycolysis and TCA cycle). Likewise, by understanding the power of the aldol reaction, one can readily recognize how the six carbon atoms of glucose might be scrambled to yield the carbon backbone of each of the 20 amino acids in a cell. When viewed through the eyes of chemical structure and reactivity, metabolic stoichiometry (and to some extent, even metabolic kinetics) are no longer "dry facts," and a rational approach can be taken toward strategic decisions regarding which reactions to manipulate toward a metabolic engineering objective.
2. *The biological logic of metabolism.* While many aspects of metabolic reaction networks are conceptually related to networks associated with other problems in chemical reaction engineering, it is the biological control of metabolism that sets this problem area apart. Concepts such as positive and negative regulation of enzyme activity at a transcriptional and/or posttranscriptional level must be understood well by chemical engineers before they can conduct a meaningful analysis of a metabolic problem. The fact that a living cell has devised a dazzling range of circuits for metabolic control, most of which have no parallel in nonliving systems, does not make the challenge any easier for the chemical engineer, but a background in molecular biology can be invaluable in this regard. The development of effective ways to translate the molecular biologist's cartoon representations of metabolic control into quantitative models was a recurrent theme in Jay Bailey's research (see, for example,

Lee and Bailey, 1984), and remains a major challenge in metabolic engineering to this day.

3. *Chemical tools.* Tools of modern analytical chemistry are especially valuable to a metabolic engineer in his analysis of perturbation-response experiments. Often, due to the highly coupled and nonlinear connections that exist within a metabolic network, it is not possible to intuitively predict the outcome of a metabolic perturbation achieved via genetic or environmental manipulation. In conjunction with steady-state or pulse-chase experiments, analytical tools such as GC/LC-MS, UV spectroscopy, fluorescence spectroscopy, and NMR spectroscopy allow one to monitor changes in concentrations and fluxes of metabolic intermediates (Bailey et al., 1987). Bailey saw the power of each one of these tools, as well as many others, and learned to not only apply them effectively to metabolic problems but also to innovate with them.
4. *Biological tools.* Above all else, the toolbox of modern molecular biology has been the driving force for the emergence of metabolic engineering science and applications. Molecular biology has provided an unparalleled ability to manipulate genes individually or combinatorially, and also to monitor structural and dynamic changes in cellular macromolecules such as DNA, RNA, and proteins. At a time when many chemical engineers were wary of the impact of molecular biology tools on their field, Bailey was one of the first chemical engineers to wholeheartedly embrace this toolbox and to put virtually every tool to use in his attack on metabolic problems. More recently, he recognized the value of genomic and proteomic tools in extending the reach of molecular biology to multi-variable analysis of metabolism.
5. *Mathematical tools.* Although metabolism is a descriptive science, metabolic engineering is most effective when undertaken on the basis of a quantitative framework. Herein lies a particularly vexing challenge—although the formalism of metabolic control theory is adequately robust to describe most problems in metabolic engineering, the complexity of most practical applications in metabolic engineering preclude rigorous application of this theory. Major (often drastic) approximations must be made that limit the generality of mathematical models. Jay Bailey experimented with the entire gamut of mathematical tools for quantitatively describing and analyzing metabolic processes, ranging from rigorous coupled nonlinear differential equations, to cybernetic models based on "soft" axioms. This was a particularly strong passion of his during his last years. His final essay titled "Complex biology with no parameters" provided advice as well as exhortation to present-day metabolic engineers (Bailey, 2001).

In addition to developing and refining metabolic engineering principles and tools, Jay Bailey also made contributions to many important problems in applied metabolic engineering. Examples of problems he studied included pH homeostasis in microorganisms, central carbon metabolism in *E. coli* and yeast, oxidative (especially microaerobic) metabolism, posttranslational modifications of proteins in mammalian cell culture, and product-oriented niche metabolism such as solventogenic fermentation processes. Within his exceptionally broad research program, each of these problems was marked by a desire to understand, control, and manipulate the metabolic property of interest. Bailey's choice of problems and his approach to a solution was characteristic, and had an enormous influence on the field. His work has been widely cited and will almost certainly be studied carefully by future generations of metabolic engineers.

DRUG DISCOVERY

Because of the complexity of many proven biologically active compounds, traditional drug discovery methods have begun to be supplemented by nontraditional methodologies that focus on either rational or combinatorial techniques for drug discovery and development. The former requires extensive knowledge about a biological target (e.g., a receptor's binding site or an enzyme's active site), and utilizes computational chemistry and molecular modeling to design chemical structures that may illicit a biological response. Often, such molecular targets are either not available, or their structures are not available in sufficient resolution to provide suitable targets for molecular modeling. In these cases, drug discovery has recently turned to combinatorial techniques for new lead discovery and development. Within the past decade there has been a major growth in the application of engineered enzymes and whole cells to problems in drug discovery. Two such examples are described below.

Combinatorial Biology

Secondary metabolism refers to metabolic processes in a cell that occur during post-exponential (often stationary) phase of growth, and are therefore unnecessary for the survival or reproductive capacity of a growing cell. Many microorganisms and plants produce structurally complex natural products as secondary metabolites; some of these have exquisite biological activities that have led to their exploitation as antibiotics, anticancer agents, or other pharmacologically useful agents. Examples of well known natural products synthesized as secondary metabolites include the antibiotics penicillin, streptomycin, erythromycin, tetracycline, vancomycin, the anticancer agents adriamycin, taxol and vinblastine, the cholesterol lowering agents compactin and lovastatin, and the immunosuppressants cyclosporin, FK506, and rapamycin.

Although it has been recognized for many decades now that, notwithstanding structural distinctions, the biosynthetic pathways of many of these natural products are related, an understanding of the associated catalytic mechanisms is only just beginning to emerge. Hand in hand with these fundamental insights, the application of protein engi-

neering principles to natural product biosynthesis has resulted in the emergence of a new field, often referred to as combinatorial biosynthesis , where the structure of a natural product is systematically manipulated by genetic manipulation of the biosynthetic enzymes. Combinatorial biosynthesis has yielded numerous new "unnatural" natural products over the past 10 years, and can be used to optimize the properties of existing and emerging bioactive natural products (Rodriguez and McDaniel, 2001). In addition, it could also be used to construct new natural product libraries for drug discovery. In particular, the enzymes responsible for biosynthesis of four major classes of natural products—polyketides, nonribosomal peptides, isoprenoids, and deoxysugars and related aminocyclitols—are emerging as especially fertile targets for genetic and chemo-biosynthetic manipulation (Cane et al., 1998). The first products from such biosynthetic engineering efforts are already entering clinical trials. As more such engineered metabolites emerge from discovery into development, the molecular vision that Jay Bailey gave to the field of biochemical engineering will undoubtedly be realized.

Combinatorial Biocatalysis for Lead Compound Optimization

Rapid developments in genomics, proteomics, and combinatorial chemistry have reshaped the field of drug discovery, providing new drug targets for selective screens and new compounds to be tested in those screens. While combinatorial methods have given rise to large libraries of compounds, typically these compounds result in improved lead candidates that must undergo further transformations by conventional medicinal chemistry to yield new drug candidates. High-throughput combinatorial methodologies have not impacted lead optimization nearly as much as they have lead discovery, mainly because of the highly selective, intricate chemistries often required to optimize lead compounds. This is particularly challenging for optimization of natural products or complex synthetic leads, the latter often coming from initial combinatorial synthesis and high-throughput screening.

Nature's most potent molecules are produced by enzyme-catalyzed reactions coupled with natural selection of those products with optimal biological activity. Combinatorial biocatalysis harnesses the natural diversity of enzymatic reactions for the synthesis of organic compound libraries to generate biologically active compounds, which encompass a wide array of chemistries and structures (Michels et al., 1998). Combinatorial biocatalysis is focused on both biocatalytic transformation and iterative synthesis. The more complex the lead compound, the more iterations that are possible and the larger the library of derivatives. Hence, thousands of derivatives of the original lead compound can be produced using combinatorial biocatalysis. Initial development of this technology focused on the generation of solution-phase combinatorial libraries, including those from synthetic precursors (e.g., dibenzyl 1,2-phenylenedioxydiacetate as bis-amide derivatives) (Adamczyk et al., 1997, as well as natural products such as flavonoids (e.g., bergenin), polyketides (e.g., doxorubicin and erythromycin) (Altreuter et al., 2002), nucleosides (e.g., adenosine), and diterpenoids (e.g., paclitaxel) (Khmelnitsky et al., 1997) have been generated in solution using enzymes or whole cells and their extracts. Together with combinatorial biology, the ability to tap into even a small part of nature's vast repertoire of biocatalytic machinery is now possible.

FINAL THOUGHTS

Jay Bailey trained as a chemical engineer during the heyday of catalytic reaction engineering as practiced in the petrochemical industry. He foresaw the impact that enzymes and cells were likely to have on the development of new products and processes, and dedicated himself to the visionary goal of educating new students of chemical engineering about the power of modern biology in this endeavor. Just a few short decades ago, not only were chemical engineers ignorant of biology, but it was difficult to make a compelling case for the relevance of biology to our discipline. The fact that catalysis and biocatalysis have merged so intimately in such a short timespan is a wonderful testament to Jay Bailey's impact on chemical engineering. Now that biology is becoming a quantitative discipline, the impact of Jay Bailey extends to the biomolecular sciences, including the interface areas of genomics and proteomics, nanobiotechnology, and high-throughput drug discovery. Thus, Bailey will impact biology in years to come as much as he impacted biochemical engineering in the past three decades. In 2050, as chemical engineers prepare to inaugurate the first zero-emission manufacturing facility that converts atmospheric carbon dioxide into automotive fuel using an engineered multienzyme system, microorganism, or plant as a catalyst, they will trace the roots of their accomplishments back to Jay Bailey's vision for molecular bioengineering in very much the same way as modern aircraft designers recognize the contributions of von Karman in the area of jet propulsion.

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Zurich, 8 July 1999

Dear Bernhard,

I look forward to seeing you again at the Biochemical Engineering Conference in Salt Lake City. In anticipation of that, I am writing with some questions about your genomics-flux balance approach.

Your recent paper in *Biotechnology Progress* is a very effective synopsis of general aspects of genomics and future challenges which I think is very useful for all bioengineers to see. The rest of the paper, presenting a number of case studies and results which are based on flux analysis, is harder for me to understand.

As I can infer from the paper, you are somehow calculating how flux distributions and specific growth rates change when certain genes are not expressed. This calculation seems to rest on an optimization calculation, which I guess must be a linear programming problem, in which the fluxes are the decision variables and making cell mass is the objective function. How does this work exactly? What is the optimization problem formulation? What are the constraints besides the metabolite mass balances? What metabolites are assumed to enter and exit the cell, and what determines those rates? And, finally, a major question, is how do you arrive at conclusions about, say, changes in specific growth rates, given a stoichiometric network? How do you get from stoichiometry to rates?

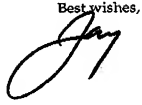
The matter of enzymes added to those implied by the genome sequence (47 of 587 according to your BP paper) is also a question. Could you please send me a list of these added steps?

I hope that you might take some time in your presentation in San Diego to explain some of these points in some detail. This method, if it is correct and if it works, is a very major advance, because it gives a formalism for determining growth rates and pathway rates without any knowledge of any kinetics. I must say it is little hard to believe that this can be done, but maybe you have made a breakthrough.

EXHIBIT 5

Any feedback you could give me before the meeting would be much appreciated. Thanks very much. See you soon.

Best wishes,



Robustness Analysis of the *Escherichia coli* Metabolic Network

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Genomic, biochemical, and strain-specific data can be assembled to define an *in silico* representation of the metabolic network for a select group of single cellular organisms. Flux-balance analysis and phenotypic phase planes derived therefrom have been developed and applied to analyze the metabolic capabilities and characteristics of *Escherichia coli* K-12. These analyses have shown the existence of seven essential reactions in the central metabolic pathways (glycolysis, pentose phosphate pathway, tricarboxylic acid cycle) for the growth in glucose minimal media. The corresponding seven gene products can be grouped into three categories: (1) pentose phosphate pathway genes, (2) three-carbon glycolytic genes, and (3) tricarboxylic acid cycle genes. Here we develop a procedure that calculates the sensitivity of optimal cellular growth to altered flux levels of these essential gene products. The results indicate that the *E. coli* metabolic network is robust with respect to the flux levels of these enzymes. The metabolic flux in the transketolase and the tricarboxylic acid cycle reactions can be reduced to 15% and 19%, respectively, of the optimal value without significantly influencing the optimal growth flux. The metabolic network also exhibited robustness with respect to the ribose-5-phosphate isomerase, and the ribose-5-phosphate isomerase flux was reduced to 28% of the optimal value without significantly effecting the optimal growth flux. The metabolic network exhibited limited robustness to the three-carbon glycolytic fluxes both increased and decreased. The development presented another dimension to the use of FBA to study the capabilities of metabolic networks.

Introduction

Genome sequencing and bioinformatics are beginning to reveal the complete set of molecular components involved in cellular activities. Furthermore, it is also clear that the integrated function of biological systems involves complex interactions among the components that have been identified through bioinformatics and genomics. Importantly, the properties of complex systems cannot be predicted simply on the basis of the complete description of their components, and the emergent properties of biological systems need to be studied (*1*, *2*). To understand the complexity inherent in cellular networks, approaches that focus on the systemic properties of the network are required. The focus of such research represents a departure from the classical reductionist approach to the integrated approach (*3*) to understanding the interrelatedness of gene function and the role of each gene in the context of multigenetic cellular functions or *genetic circuits* (*4*, *5*).

The engineering approach to analysis and design is to have a mathematical or computer model, e.g., a dynamic simulator, of a cellular process that is based on fundamental physicochemical laws and principles. There has been a long history of mathematical modeling of metabolic systems, which dates back to the to the mid 1960s. With the availability of analogue computers and the knowledge of metabolic regulation, dynamic simulations of simple metabolic and genetic control loops appeared (*6*). The dynamic stability of such control loops became a focus of attention (*7*, *8*), given the experimental observations of oscillatory dynamics in yeast glycolysis (*9*).

The systemic nature of metabolic function was apparent, and so was its complexity. However, the availability of enzyme kinetic information was fragmented, and attention turned to developing methods that could shed light on the relative importance of various metabolic events. Methods for sensitivity analysis of metabolic regulation began in the 1960s (*10*) and continued into the 1970s (*11*, *12*). The results of these undertakings were biochemical systems theory (BST) and metabolic control analysis (MCA), and some useful results have been obtained using these approaches (*13*).

Establishing complete kinetic models of cellular metabolism became a scientific goal, whose intended use was to elucidate the systemic behavior of metabolic networks. Because of its simplicity, the human red blood cell represented the best opportunity to achieve this goal. Early metabolic models of human red blood cell metabolism appeared in the 1970s (*11*) and continued throughout the 1980s and 1990s (*14*–*16*). Insights into the functioning of this cell have resulted from these analyses (*11*, *17*, *18*). Although interesting in their own right, studies of red cell metabolism are not directly useful for organisms of industrial importance.

While the ultimate goal is the development of dynamic models for the complete simulation of metabolic systems, the success of such approaches has been severely hampered by the current lack of kinetic information on the dynamics and regulation of metabolic reactions. However,

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in the absence of kinetic information it is still possible to accurately assess the theoretical capabilities and operative modes of metabolic systems using metabolic flux balance analysis (FBA) (*5, 19–23*). FBA is based on the fundamental physicochemical constraints on metabolic networks. FBA only requires information regarding the stoichiometry of metabolic pathways and the metabolic demands; furthermore, FBA can incorporate additional information when it is available. FBA is particularly applicable for post-genomic analysis, because the stoichiometric parameters can be defined from the annotated genome sequence (*21*).

In a previous article, we have examined the capability of *in silico* mutant *E. coli* metabolic networks to support growth and compared the results to the wildtype. By using computer simulations, it was determined that seven metabolic reactions were essential for the aerobic growth of *E. coli* in glucose minimal media (*24*). The remaining reactions were determined to be nonessential, since the metabolic network maintained the capability to bypass simulated metabolic defects, often with little or no effect on the *in silico* maximal biomass yield. In this article we will further examine the essential metabolic reactions by examining the metabolic consequences of reduced metabolic flux carrying capacity in the essential reactions. The results indicate the redundancy and robustness in the function of the respective metabolic reactions in the metabolic network by examining the sensitivity of the objective function to the quantitative flux levels. The sensitivity analysis can provide information regarding the experimental measurements that are likely to provide the most information toward quantitatively describing the metabolic network and can be used for *in silico* experimental design and assessing the value of the *in silico* predictions.

Describing Metabolic Systems

A metabolic network is a collection of enzymatic reactions that serve to biochemically process metabolites within the cell and transport processes that convert extracellular metabolites to intracellular metabolites and vice versa. To quantitatively describe metabolic networks, dynamic mass balances are written for each metabolite in the network, generating a system of ordinary differential equations that describe the transient behavior of metabolite concentrations:

$$\frac{dX_i}{dt} = \sum_j S_{ij} v_j \qquad (1)$$

where v_j corresponds to the jth metabolic flux, X_i represents the ith metabolite, and the stoichiometric coefficient S_{ij} stands for the number of moles of metabolite i formed (or consumed) in reaction j. Equation 1 is particularly difficult to solve since the metabolic fluxes are often nonlinear functions of the metabolite concentrations, as well as a set of kinetic parameters that are difficult to measure or estimate. The complexity associated with estimating the functional relation between the metabolic fluxes and the metabolite concentrations and the associated kinetic parameters has hampered the quantitative analysis of metabolic networks.

Constraining Metabolic Functions

Given the complexities associated with quantitative analysis of metabolic systems based on kinetic characterization of the components, we have utilized a conceptually different approach to the analysis of metabolic networks. First, we defined fundamental physicochemical constraints to which the metabolic network is constrained. Then, the metabolic capabilities were assessed subject to the imposed constraints. The capabilities are analyzed under the steady state assumption. It should be noted that steady state analysis is applicable to some aspects of metabolism; however, the approach will not be appropriate for studying all cellular processes, such as the cell cycle or signal transduction. Herein, we are interested in metabolic processes and their relation to cellular growth; thus the characteristic time of the processes is about an hour. Metabolic transients within the cell typically occur with time constants on the order of seconds to minutes (*25*); thus under our "window of observation" the metabolic network is essentially in a steady state and the steady-state analysis will be appropriate. The steady-state mass, energy, and redox balance constraints are imposed by simplifying eq 1:

$$\mathbf{S} \cdot \mathbf{v} = 0 \qquad (2)$$

where $\mathbf{S}$ is the stoichiometric matrix and $\mathbf{v}$ is the flux vector. While the system is closed to the passage of certain metabolites, others are allowed to enter or exit the system via exchange fluxes (or pseudoreactions (*26*)). These fluxes do not represent biochemical conversions or transport processes such as those of internal fluxes but can be thought of as representing the inputs and outputs to the system. For example, the demand on a metabolite for further processing or incorporation into cellular biomass creates an exchange flux on the internal cellular metabolite. Thus, a distinction is made between internal and external metabolites in the system, therefore closing the material balance to all metabolites as indicated by eq 2.

To complete the *in silico* representation of the metabolic network we included the constraints on the individual metabolic reaction fluxes due to reaction thermodynamics and the input/output characteristics of the network. All reversible metabolic reactions were assumed to have the capability to carry any metabolic flux (i.e., $-\infty \leq v_j \leq \infty$; where v_j is the flux in reversible reactions), whereas irreversible metabolic reactions fluxes were restricted to be positive (i.e., $0 \geq v_j \geq \infty$; where v_j is the flux in irreversible reactions). Although constraints on the internal fluxes were defined as infinite, the magnitude of each flux in the optimal solution was examined and compared to measured fluxes (*27, 28*). The reversibility of each reaction in the metabolic network was determined case by case on the basis of the literature and compared to the EcoCyc database (*29*). The metabolic enzymes identified in the complete *E. coli* K12 genome sequence and the online databases (*29–31*) were used to reconstruct the metabolic network (see supplementary information at http://gcrg.ucsd.edu/supplementary_data/BP2000/main.htm). It should be noted that there are instances where the same enzyme can catalyze multiple reactions (e.g., different substrates or cofactors), and this situation was considered by including all reactions catalyzed by an enzyme as a separate column in the stoichiometric matrix. The details of this metabolic reconstruction have been described elsewhere (*24*). Additionally, constraints were placed on the exchange fluxes to indicate the environmental conditions. For example, metabolites not available to the cell are constrained to not enter the cell: $-\infty \leq b_i \leq 0$, where b_i (influx defined as positive) is the exchange flux for a metabolite not available in the simulated environment. It should be noted that all metabolites that have the capability to leave the cell always had unconstrained metabolic fluxes

in the net outward direction, whereas the influx constraints were defined by the simulated environmental conditions. For the analysis herein, the exchange flux for inorganic phosphate, ammonia, carbon dioxide, oxygen, sulfate, potassium, and sodium were unconstrained, whereas the uptake of the carbon source was constrained as specified.

Demands on the Metabolic Network

Under changing substrate/supply conditions metabolic networks are continuously faced with a balanced set of biosynthetic demands (i.e., production of amino acids, nucleotides, phospholipids, as well as energy and redox potential). Effectively this means that the network must generate a balanced set of metabolites that are used to produce biomass. The biosynthetic demands for growth were determined from the biomass composition of *E. coli* (*32, 33*), and a metabolic flux, defined as the growth flux (v_{growth}), utilizes the biosynthetic precursors in the appropriate ratios so as to generate biomass:

$$\sum_{i}^{m} d_i \cdot X_i \xrightarrow{v_{growth}} \text{Biomass}$$

where d_i (mmol · g-dry weight $(DW)^{-1}$) is the *E. coli* biomass composition of metabolite *i*. One gram of biomass is produced per unit flux in the growth flux, v_{growth}, and if the fluxes are represented with a basis of 1 g-DW · h (*22*), the growth flux is equivalent to the growth rate. The biomass composition is not constant but depends on the growth rate and the growth conditions (*33*). However, we have assumed that the biomass composition is constant since it has been shown that the optimal solution is not sensitive to the biomass composition (*34*), and this observation is also true for our system.

In addition to the biosynthetic demands on the metabolic network, we have also imposed maintenance requirements on the metabolic system. The maintenance requirements included were for growth-associated and non-growth-associated maintenance. We imposed a growth-associated maintenance of 23 mmol ATP · g-DW^{-1} and a non-growth-associated maintenance of 5.87 mmol ATP · g-DW^{-1} · h^{-1} (*35*).

Exploring the Metabolic Capabilities

The constraints on the metabolic network define the boundaries within which the metabolic system must operate. The mass, energy, and redox balance constraints are imposed by the linear homogeneous set of equations (eq 2). The nullspace of the stoichiometric matrix, **S**, contains all flux vectors that satisfy the mass, energy, and redox balance constraints (*36*). However, there are additional physicochemical constraints on the metabolic network, such as the thermodynamic constraints and the capacity constraints on the exchange fluxes, which are enforced by linear inequalities. The simultaneous enforcement of all the metabolic constraints defines a region, the *feasible set*, that contains all feasible metabolic flux vectors. The feasible set is not a vector space as is the nullspace, as a result of the linear inequality constraints. Importantly, the feasible set defines the metabolic capabilities of the system. The performance capabilities of any metabolic network reside in the feasible set. In fact, the answer to any question related to the general structure and fitness of the network lies with this region. While the feasible set offers a convenient way of defining metabolic capabilities, the question arises, how do we best explore the specific functions of a metabolic network?

One approach that has been used to explore the relationship between the metabolic genotype and phenotype for a number of organisms is linear optimization (*19, 21, 22, 37*). Linear optimization was used to determine the optimal flux distributions within a network so as to maximize/minimize a particular objective function. A linear programming problem is defined as follows, where a linear objective function is maximized or minimized subject to a series of linear equality and inequality constraints:

$$\text{Maximize/Minimize } Z = c_j v_j$$
$$\text{subject to } S_{ij} v_j = 0,\ \alpha_j \le v_j \le \beta_j \qquad (5)$$

The linear programming formalism is analogous to the system of linear equalities/inequalities that form the constraints on the metabolic network. The objective function, *Z*, is defined by assigning the appropriate values to the **c** vector; herein, the **c** vector was taken as the unit vector in the direction of the growth flux. We used the reduced costs from the linear programming solution to identify alternate optimal solutions. In metabolic engineering applications, the objective function can correspond to a number of diverse objectives, such as maximizing energy or metabolite production (*20*). However, regardless of the objective function the optimal solution will lie within the feasible set that is defined by the physicochemical constraints placed on the system.

The utilization of linear programming to examine metabolic networks defines the optimal flux vector that maximizes (or minimizes) an objective function and satisfies the entire set of constraints. The utilization of design related objectives (such as maximizing the production of an amino acid) can be used to guide genetic engineering of a strain for metabolite overproduction. Herein, we have employed a physiologically realistic objective, the maximization of the growth flux. We have assumed that the cell has evolved the regulatory mechanisms to operate optimally within the feasible set. The feasible set defines the capabilities of the metabolic network, and all metabolic flux vectors within the feasible set satisfy the imposed physicochemical constraints. Therefore, theoretically all flux vectors within the feasible set can be reached by adjusting the enzyme kinetic parameters and gene regulation. The enzyme kinetics and gene regulation constraints on the metabolic system will be referred to as system specific constraints. We assume that the cell has found the optimal set of system specific constraints through the course of evolution, and we attempt to find the same solution using linear programming. The assumption has been experimentally examined under a limited number of conditions, and under defined conditions with a single carbon source, the experimental data is consistent with the optimal utilization of the metabolic network (*27*).

Phenotype Phase Plane Analysis

Flux balance analysis can be used to examine the metabolic network in detail. Optimal solutions to the linear programming problem will then lie on a vertex of the feasible set, which is a polyhedron (*38*). All the metabolic flux vectors (or metabolic phenotypes) attainable from a defined metabolic genotype are mathematically confined to the feasible set. Linear programming was used to search through the feasible set for a solution that maximizes the growth flux. Experimental data for the growth of *E. coli* under nutritionally rich growth conditions (i.e., cell is not starved for phosphate, nitrogen,

etc.) is consistent with the optimal utilization of the metabolic network (*27*); thus, defining the growth flux as the objective function produces physiologically meaningful results. However, the optimal flux distribution is only meaningful when interpreted in terms of the specific environmental conditions. Therefore, phenotype phase planes (*39*) have been developed to define the range of optimal flux vectors and how the optimal flux vector is dependent on the environmental conditions.

The methodology for defining PhPPs has been described (*39*). We will now briefly describe the construction of PhPPs. Two metabolic fluxes can form two axes on an (x,y)-plane (these metabolic fluxes were two unit vectors in R^n). The optimal metabolic flux distribution is calculated for all points in this plane. In other words, the maximum value of the objective function is found as the position of the hyperplanes that bound the feasible set in the respective directions is moved. It has been determined that there are a finite number of fundamentally different optimal metabolic flux distributions (or basis solutions in linear programming terminology) present in such a plane. The demarcations on the phase plane were defined by a shadow price (LP dual variable) analysis (*40*). This procedure leads to the definition of distinct regions, or "phases", in the plane, in which the optimal use of the metabolic network is fundamentally different, corresponding to different optimal phenotypes.

Robustness Analysis

Robustness, defined here with respect to metabolic networks, is a measure of the change in the maximal flux of the objective function (the growth flux was defined as the objective) when the optimal flux through any particular metabolic reaction is changed. The robustness characteristics of the metabolic network were determined by calculating the optimal flux vector so as to maximize the growth flux (with only external flux constraints), this flux was called the *in silico* wildtype flux. Then the flux through the reaction in question was reduced from 100% to 0% of the *in silico* wildtype flux and the objective function was calculated. Additionally, the *in silico* wildtype flux was increased from the wildtype value and the upper bound on increasing the flux level was the maximal allowable flux in the reaction or the flux level for which the objective function was reduced to zero. The calculations were for a simulated aerobic batch culture in glucose minimal media.

The FBA framework was used to address the systemic effect on the metabolic network of increased and decreased (with respect to the *in silico* wildtype) metabolic flux. Herein, we quantified the robustness of the metabolic network to flux changes in the essential enzymatic reactions. The essential enzymes (for growth on glucose minimal media) were previously identified through an *in silico* analysis (*24*). Seven enzymatic reactions in central metabolism (Figure 1) were found to be essential: the transketolase (TKT), ribose-5-phosphate isomerase (RPI), two enzymes (GAP, PGK) in the 3-carbon stage of glycolysis (3CG), and the first three enzymes (GLT, ACN, ICD) of the TCA cycle. Below, the robustness characteristics of the metabolic network with respect to alterations of the flux levels of these essential metabolic reactions will be investigated. We will utilize phenotype phase planes (PhPPs) to define points where the optimal utilization of the metabolic network changes due to capacity constraints on the essential enzymatic reactions.

Transketolase. The transketolase (TKT) catalyzes an essential enzymatic reaction in the pentose phosphate pathway (PPP) (*41*). However, *tkt* mutant strains have been shown to grow on glucose minimal media with low TKT residual activity (3% of wildtype) (*42*, *43*). The ability of the metabolic network to support growth with a large reduction in TKT flux was investigated *in silico* by continuously restricting the metabolic flux in the TKT reactions. As the maximum allowable flux through the TKT reactions was reduced from the *in silico* wildtype, it was determined that the ability of the metabolic network to support growth was virtually unchanged for enzymatic fluxes as low as 15% of the *in silico* wildtype (Figure 2). The response to decreased TKT metabolic flux was found to have two qualitatively different regions. The regions were identified in the PhPP (Figure 3).

The PhPP describing the changes in the metabolic pathway utilization as a function of the TKT flux and the glucose uptake rate was calculated (Figure 3). The optimal relation between the glucose uptake rate and the TKT flux was determined from the PhPP (Figure 3). It was determined that there were two qualitatively different regions of metabolic pathway utilization for TKT fluxes lower than optimal, and these regions were defined as A and B (as shown in Figure 3). Furthermore, there were determined to be six qualitatively different regions for TKT fluxes greater than optimal, and these regions were defined as 1–6 (as shown in Figure 3). The maximal growth flux (normalized to the *in silico* wildtype) was calculated for all TKT fluxes from zero to the maximum allowable flux (the glucose uptake exchange flux was constrained to 10 mmol g-DW^{-1} h^{-1}) that still permits cellular growth, and the results are shown in Figure 2.

In region A (above 15% of the *in silico* wildtype enzyme flux), the optimal value of the growth flux was hardly changed, and at the demarcation between regions A and B, the growth flux was decreased to 99.2% of the *in silico* wildtype. However, to cope with the decreased TKT metabolic flux carrying capacity, shifts in the metabolic pathway utilization occurred (Figure 4). The redox potential (NADPH) requirement for biosynthetic demands was achieved by a flux redistribution that resulted in the utilization of the transhydrogenase that converted NADH (produced from an increased TCA cycle flux, Figure 4C) into NADPH. The flux diverted from the PPP (Figure 3B) resulted in increased glycolytic fluxes, in particular the pyruvate kinase and the phosphoglucoisomerase fluxes (Figure 3A). In this region, the optimal growth flux was not sensitive to changes in the TKT flux. However, the optimal flux in several metabolic processes were sensitive to the TKT flux in this region (transhydrogenase, PYK, PGI, TCA cycle flux)

In the second region (region B) of reduced TKT flux (enzyme flux less than 15% of the *in silico* wildtype), the metabolic network was limited in the ability to produce the essential biosynthetic precursor, erythrose 4-phosphate. In this region, the optimal growth flux was sensitive to the flux level in the TKT reaction. The metabolic fluxes in this region are not shown in Figure 4 because alternate optimal solutions exist. Cellular growth is solely limited by the availability of a single biosynthetic precursor, the excess glucose can be converted to any of the metabolic byproducts with the same value of the objective function. Furthermore, the excess high-energy phosphate bonds can be eliminated in any futile cycle; thus alternate optimal solutions exist.

The effect on the metabolic network due to TKT fluxes increased beyond the optimal flux for growth was also examined. An increase in metabolic flux may result from the overexpression of the respective gene, and the robustness analysis can be used to identify the constraints on flux changes due to the integrated metabolic network.

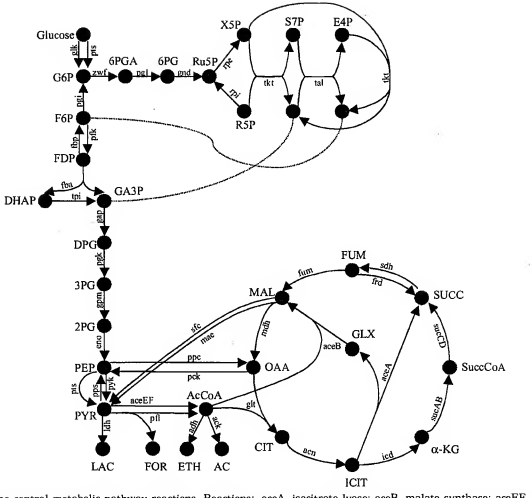

Figure 1. The central metabolic pathway reactions. Reactions: aceA, isocitrate lyase; aceB, malate synthase; aceEF, pyruvate dehydrogenase; ack, acetate kinase; acn, aconitase; adh, acetaldehyde dehydrogenase; eno, enolase; fba, fructose-1,6-bisphosphatate aldolase; fbp, fructose-1,6-bisphosphatase; frd, fumarate reductase; fum, fumarase; gap, glyceraldehyde-3-phosphate dehydrogenase; glk, glucokinase; glt, citrate synthase; gnd, 6-phosphogluconate dehydrogenase; gpm, phosphoglycerate mutase; icd, isocitrate dehydrogenase; ldh, lactate dehydrogenase; mae, malic enzyme; mdh, malate dehydrogenase; pck, phosphoenolpyruvate carboxykinase; pfk, phosphofructokinase; pfl, pyruvate formate lyase; pgi, phosphoglucose isomerase; pgk, phosphoglycerate kinase; pgl, 6-phosphogluconolactonase; ppc, phosphoenolpyruvate carboxylase; pps, phosphoenolpyruvate synthase; pts, phosphotransferase system; pyk, pyruvate kinase; rpe, ribulose phosphate 3-epimerase; rpi, ribose-5-phosphate isomerase; sdh, succinate dehydrogenase; sfc, malic enzyme; sucAB, 2-ketoglutarate dehyrogenase; sucCD, succinyl-CoA synthetase; tal, transaldolase; tkt, transketolase; tpi, triosphosphate isomerase; zwf, glucose 6-phosphate-1-dehydrogenase. Metabolites: 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, D-6-phosphate-gluconate; 6PGA, D-6-phosphate-glucono-δ-lactone; AC, acetate; AcCoA, Acetyl-CoA; α-KG, α -ketoglutarate; CIT, citrate; DHAP, dihydroxyacetone phosphate; DPG, 1,3-bis-phosphoglycerate; E4P, erythrose 4-phosphate; ETH, ethanol; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; FOR, formate; FUM, fumarate; G6P, glucose 6-phosphate; GA3P, glyceraldehyde 3-phosphate; ICIT, isocitrate; LAC, lactate; MAL, malate; PEP, phosphoenolpyruvate; PYR, pyruvate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedo-heptulose; SUCC, succinate; SuccCoA, succinyl CoA; X5P, dihydroxyacetone phosphate.

The optimal growth flux as a function of the flux in the TKT reaction was calculated (Figure 2, insert). The metabolic flux was continuously increased *in silico* from the *in silico* wildtype value to the maximum flux that permits growth. Six qualitatively different patterns of metabolic pathway utilization (numbered 1–6 in Figure 3) were observed when the TKT flux was increased beyond the *in silico* wildtype.

The region 1 of Figure 3 the optimal metabolic flux vector was characterized by an increased PPP flux, an active transhydrogenase reaction, a decreased PYK flux, and a decreased TCA cycle flux. In region 2 the TCA cycle flux was further decreased while the PPP flux was increased, and optimally, the glyoxylate bypass was utilized to replenish the TCA cycle biosynthetic precursors thus reducing the PPC flux (Figure 4). At the demarcation between regions 2 and 3, the TCA cycle was shut off and functioned to produce the biosynthetic precursors, rather than redox potential. Optimally, in region 3, the glucokinase reaction was operative in glucose utilization, and this allowed for a more efficient flow of the metabolites into the PPP due to the increased TKT flux. Regions 4 and 5 were similar with respect to the metabolic pathway utilization, the glyoxylate bypass was no longer utilized in the optimal solution and the PFL reaction optimally carried a small flux. Finally, in region 6, redox potential was overproduced. This region was characterized by alternate optimal solutions to eliminate the excess high-energy phosphate bonds. However, there were no metabolic byproducts produced (other than CO_2); this was because many metabolites were still desirable to the cell (as identified through a shadow price analysis (*40*)). In this region, the optimal oxygen uptake rate was very high (~60 mmol g-DW^{-1} h^{-1}) and it is likely that the maximal TKT flux is much lower due to other constraints on the metabolic network that were not

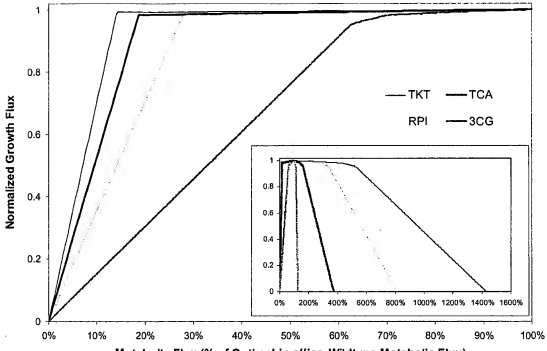

Figure 2. Robustness diagrams. The effect of altered metabolic flux in the essential metabolic reactions on the normalized growth flux is illustrated. The *in silico* wildtype flux is defined as 100%. See the text for a complete discussion.

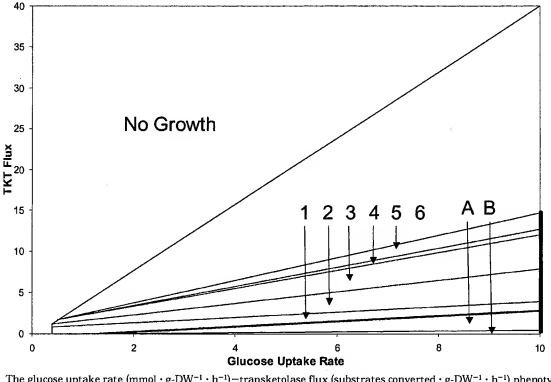

Figure 3. The glucose uptake rate (mmol · g-DW^{-1} · h^{-1})−transketolase flux (substrates converted · g-DW^{-1} · h^{-1}) phenotype phase plane. Exchange flux constraints were defined as discussed in the text. The regions are numbered and lettered. The numbered regions correspond to TKT fluxes that are increased relative to the optimal value. The optimal relation is the thick demarcation line. The lettered regions identify TKT flux reductions below the optimal relation. The metabolic fluxes along the thick vertical line (glucose uptake rate = 10 mmol · g-DW^{-1} · h^{-1}) are shown in Figure 4.

included in the analysis (such as oxygen mass transfer limitations).

Ribose-5-Phosphate Isomerase. The ribose-5-phosphate isomerase reaction (RPI) also catalyzes an essential reaction in the PPP (*41*) for growth in glucose minimal media. Similarly to *tkt* mutants, *rpi* mutants have been shown to grow with enzymatic activity much less than that of the wildtype. For example, Skinner and Cooper have isolated a strain with RPI activity below 10% of the wildtype, and this strain was able to grow (*44*). As the

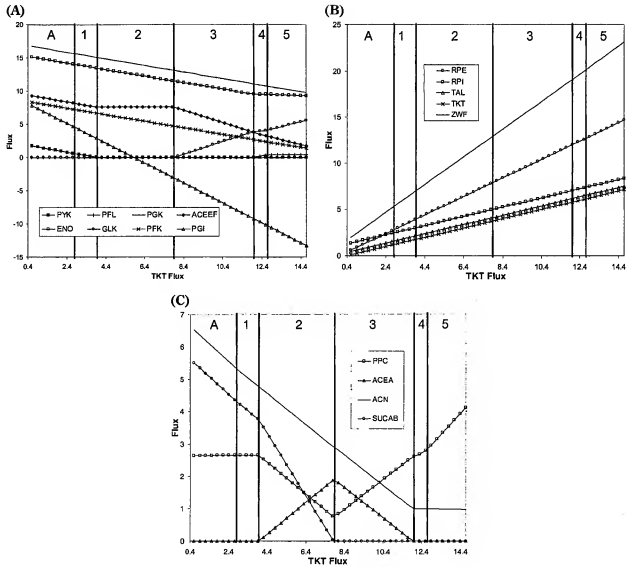

Figure 4. Optimal intracellular fluxes in the central metabolic pathways (substrates converted · g-DW^{-1} · h^{-1}) as a function of the TKT metabolic flux constraint (substrates converted · g-DW^{-1} · h^{-1}). The glucose uptake rate was constrained to 10 mmol · g-DW^{-1} · h^{-1}. (A) Glycolytic fluxes. (B) Pentose phosphate pathway fluxes. (C) TCA cycle fluxes.

maximum allowable RPI flux was reduced from the *in silico* wildtype, it was determined that the ability of the metabolic network to support growth was virtually unchanged for enzymatic fluxes as low as 28% of the *in silico* wildtype (Figure 2). The RPI-glucose uptake rate PhPP was calculated to characterize the effect of altered RPI metabolic fluxes (not shown, similar to Figure 3). The qualitative effect of reduced flux in the RPI reaction from the *in silico* wildtype was investigated with FBA, and the holistic metabolic response to decreased and increased RPI fluxes was similar to the TKT results because the effect on the PPP was similar.

3-Carbon Glycolysis. The 3-carbon glycolytic reactions that the *in silico* analysis predicted to be essential have been shown experimentally to be required for the growth of *E. coli* on a glucose minimal media (GAP, PGK) (*41*). The glycolytic essential reactions were subjected to a robustness analysis to investigate the optimal systemic effect of flux alteration. The ability of the metabolic network to support growth with a reduction in 3CG flux was investigated *in silico* by continuously restricting the 3CG flux. As the allowable flux through the 3CG reactions was reduced from the *in silico* wildtype, it was determined that the sensitivity of the growth flux was increased compared to the other essential reactions. When the 3CG flux was reduced below about 70% of the *in silico* wildtype, the growth flux was sensitive to the 3CG flux (Figure 2). Furthermore, the 3CG fluxes could only be increased to 110% of the *in silico* wildtype before severe limitations in the growth flux were encountered (Figure 2). We have investigated the metabolic response to 3CG flux level alterations by a phenotype phase plane analysis (Figure 5).

The PhPP describing the changes in the metabolic pathway utilization as a function of the 3CG flux and the glucose uptake rate was calculated (Figure 5). The optimal relation between the glucose uptake rate and the 3CG flux was determined from the PhPP (Figure 5). It was determined that there were six qualitatively different regions of metabolic pathway utilization for 3CG fluxes lower than optimal, and these regions were defined as A−F (as shown in Figure 5). Furthermore, there were determined to be two qualitatively different regions for 3CG fluxes greater than optimal, and these regions were

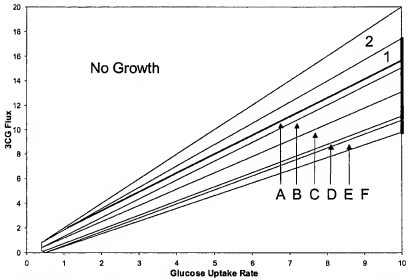

Figure 5. The glucose uptake rate (mmol · g-DW^{-1} · h^{-1})–3-carbon glycolytic flux (substrates converted · g-DW^{-1} · h^{-1}) phenotype phase plane. Exchange flux constraints were defined as discussed in the text. The regions are numbered and lettered. The numbered regions correspond to 3CG fluxes that are increased relative to the optimal value. The optimal relation is the thick demarcation line. The lettered regions identify 3CG flux reductions below the optimal relation. The metabolic fluxes along the thick line (glucose uptake rate = 10 mmol · g-DW^{-1} · h^{-1}) are shown in Figure 6.

defined as 1–2 (as shown in Figure 5). The maximal growth flux (normalized to the *in silico* wildtype) was calculated for all 3CG fluxes from zero to the maximum allowable flux (the glucose uptake exchange flux was constrained to 10 mmol g-DW^{-1} h^{-1}) that still permits cellular growth, and the results are shown in Figure 2.

The optimal relation between the glucose uptake and the 3CG flux was calculated (Figure 5). The sensitivity of other optimal fluxes in the metabolic network upon the reduction of the 3CG flux was examined (Figure 6). With 3CG flux reduction just below the optimal value, the optimal metabolic network operation was characterized by region A (Figure 5). In region A, the 3CG flux reduction led to increased PPP fluxes and the transhydrogenase was used (Figure 6B); additionally, the TCA cycle flux was reduced (Figure 6C). The reduced 3CG flux also led to the reduction of the PYK flux, which was optimally completely inactivated at the demarcation between regions A and B (Figure 6A). In region B, the glyoxylate bypass was optimally utilized and the TCA cycle fluxes were further reduced. At the demarcation between region B and C, the TCA cycle no longer operated cyclically but rather served to generate the biosynthetic precursors. In region C, glucokinase was included in the optimal flux vector, the inclusion of the glucokinase decoupled the phosphoenolpyruvate to pyruvate biochemical conversion and the uptake of glucose, thus allowing for the 3CG flux to be decreased and with little effect on the maximal growth flux. The growth flux at the demarcation between region C and D was 98% of the *in silico* wildtype (Figure 2).

Regions D and E were very similar with respect to the optimal metabolic flux vector. In these regions, the pyruvate–formate lyase was optimally active and the glyoxylate bypass was no longer included in the optimal flux vector. Additionally, in regions D and E, the growth flux was more sensitive (compared to regions A–C) to the 3CG flux, and at the demarcation between regions E and F the maximal growth flux was 95% of the *in silico* wildtype.

In the final region of reduced 3CG flux (region F, metabolic flux less than 63% of the *in silico* wildtype), the metabolic network was limited in the ability to produce the essential biosynthetic precursors below the block in the metabolic network. In this region, the optimal growth flux was sensitive to the 3CG flux, and the maximal growth flux linearly decreased to zero as the 3CG flux was reduced to zero from the region E,F boundary. The metabolic fluxes in this region are not shown in Figure 6 because alternate optimal solutions exist. Cellular growth is limited by the availability of the biosynthetic precursors after the metabolic blockage, and the diversion of the flux from glycolysis to the PPP resulted in excess high-energy phosphate bonds and redox potential. The growth flux in region F is dependent upon increased oxygen availability to eliminate the excess redox potential. An additional constraint was imposed on the metabolic network, i.e., the oxygen uptake was constrained below a physiologically realistic value of 20 mmol g-DW^{-1} h^{-1}, and the feasible set did not contain a growth flux for 3CG fluxes below about 40% of the *in silico* wildtype (not shown). Thus, the partial inhibition of the 3CG fluxes can theoretically prevent the growth of *E. coli*; however, growth can be maintained with reduced glucose uptake rates.

The holistic effect of increased 3CG flux on the metabolic networks capability to support cellular growth was assessed with FBA. The optimal growth flux as a function of the flux in the 3CG flux was calculated (Figure 2). The metabolic flux was continuously increased *in silico* from the *in silico* wildtype value to the maximum flux that permits growth, and two qualitatively different metabolic flux vectors (numbered 1 and 2 in Figure 5) were observed.

In region 1 (Figure 5), the optimal metabolic flux vector was characterized by a decreased pentose phosphate pathway (PPP), an active transhydrogenase reaction, an increased PYK flux, and an increased TCA cycle flux. However, region 1 only extends to a 3CG flux of 110% of the *in silico* wildtype (with a glucose uptake of 10 mmol g-DW^{-1} h^{-1}), and region 2 of Figure 5 was characterized by alternate optimal flux distributions. The metabolic network was limited in the ability to produce the essential biosynthetic precursors before the effected reac-

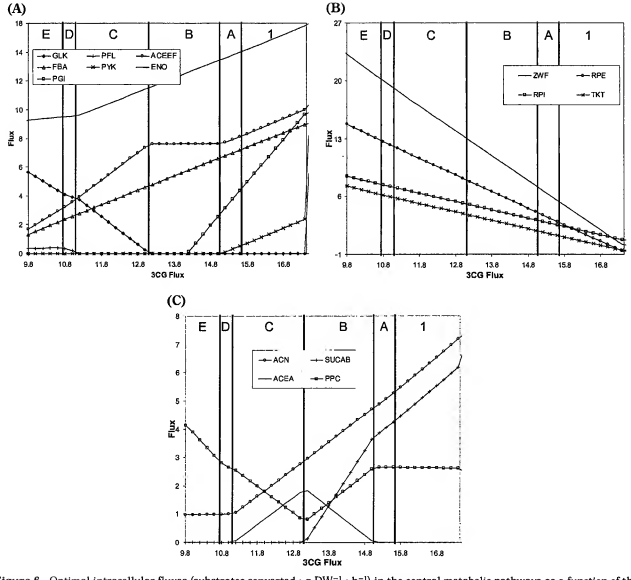

Figure 6. Optimal intracellular fluxes (substrates converted · g-DW^{-1} · h^{-1}) in the central metabolic pathways as a function of the 3CG metabolic flux constraint (substrates converted · g-DW^{-1} · h^{-1}). The glucose uptake rate was constrained to 10 mmol g-DW^{-1} h^{-1}. (A) Glycolytic fluxes. (B) Pentose phosphate pathway fluxes. (C) TCA cycle fluxes.

tions of 3-carbon glycolysis. In this region, the optimal growth flux was sensitive to the 3CG flux. The metabolic fluxes in this region are not shown in Figure 6 because alternate optimal solutions exist. The excess pyruvate produced by the elevated 3CG flux can be converted to any of the metabolic byproducts with the same value of the objective function. Furthermore, the excess high-energy phosphate bonds can be eliminated in any futile cycle, and thus alternate optimal solutions exist.

TCA Cycle. The initial three fluxes of the TCA cycle were determined to be essential. The deletion of any of these enzymatic activities resulted in a glutamate requirement. This requirement has been shown experimentally (*45*). The *in silico* robustness analysis was performed to assess the effect of decreased (and increased) flux entering the TCA cycle (Figures 2, 7, and 8). The ability of the metabolic network to support growth with a reduction in the TCA cycle flux was investigated *in silico* by continuously restricting the citrate synthase flux, which we will refer to as the TCA cycle flux. As the TCA cycle flux constraint was reduced from the *in silico* wildtype, it was determined that the ability of the metabolic network to support growth was not sensitive to the TCA cycle flux above 18% of the *in silico* wildtype. Furthermore, the TCA cycle flux could be increased to about 160% of the *in silico* wildtype before severe limitations in the growth flux were encountered (Figure 2). We have investigated the metabolic response to TCA cycle flux level alterations by a phenotype phase plane analysis (Figure 7).

The PhPP describing the changes in the metabolic pathway utilization as a function of the TCA cycle flux and the glucose uptake rate was calculated (Figure 7). The optimal relation between the glucose uptake rate and the TCA cycle flux was determined from the PhPP (Figure 7). It was determined that there were four qualitatively different regions of optimal metabolic pathway utilization for TCA cycle fluxes lower than optimal, and these regions were defined as A–D (as shown in Figure 7). Furthermore, there were determined to be four qualitatively different regions for TCA cycle fluxes greater than optimal, and these regions were defined as 1–4 (as shown in Figure 7). The maximal growth flux (normalized to the *in silico* wildtype) was calculated for all TCA cycle

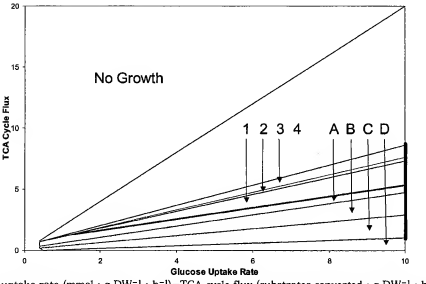

Figure 7. The glucose uptake rate (mmol · g-DW^{-1} · h^{-1})–TCA cycle flux (substrates converted · g-DW^{-1} · h^{-1}) phenotype phase plane. Exchange flux constraints were defined as discussed in the text. The regions are numbered and lettered. The numbered regions correspond to TCA cycle fluxes that are increased relative to the optimal value. The optimal relation is the thick demarcation line. The lettered regions identify TCA cycle flux reductions below the optimal relation. The metabolic fluxes along the thick line (glucose uptake rate = 10 mmol · g-DW^{-1} · h^{-1}) are shown in Figure 8.

fluxes from zero to the maximum allowable flux (The glucose uptake exchange flux was constrained to 10 mmol g-DW^{-1} h^{-1}) that still permits cellular growth, and the results are shown in Figure 2.

The optimal relation between the glucose uptake and the TCA cycle flux was calculated, and the sensitivity of the optimal fluxes in central metabolism to the TCA cycle flux was examined (Figure 8). Region A defines the optimal set of metabolic reactions that are utilized with a reduction of the TCA cycle flux below the optimal value (Figure 7). In region A, the reduction of the TCA cycle flux led to increased PPP fluxes and the transhydrogenase was used; additionally, the glycolytic flux was decreased. The reduced TCA cycle flux led to the reduction of the PYK flux, which was optimally completely inactivated at the demarcation between regions A and B. In region B, the glyoxylate bypass was optimally utilized and the glycolytic fluxes were further reduced. At the demarcation between region B and C, the TCA cycle fluxes were reduced to the point that the TCA cycle no longer operated cyclically but rather served to generate the biosynthetic precursors. In region C, glucokinase was included in the optimal flux vector, and the inclusion of the glucokinase decoupled the phosphoenolpyruvate to pyruvate biochemical conversion and the uptake of glucose. The growth flux at the demarcation between region C and D was 98% of the *in silico* wildtype (Figure 2) and the TCA cycle flux was 18% of the *in silico* wildtype TCA cycle flux.

In the final region of reduced TCA cycle fluxes (metabolic flux less than 18% of the *in silico* wildtype), the metabolic network was limited in the ability to produce α-ketoglutarate, an essential biosynthetic precursor. In this region, the optimal growth flux was sensitive to the TCA cycle flux, and the characteristic behavior was similar to region F of the 3CG-glucose uptake rate PhPP (Figure 5) that was discussed above.

The holistic effect of increased TCA cycle flux on the metabolic networks' capability to support cellular growth was assessed with FBA. The optimal growth flux as a function of the flux in the TCA cycle flux was calculated (Figure 2). The metabolic flux was continuously increased *in silico* from the *in silico* wildtype value to the maximum flux that permits growth, and four qualitatively different metabolic flux vectors (numbered 1–4 in Figure 7) were observed.

The region 1 of Figure 7 the optimal metabolic flux vector was characterized by an active transhydrogenase reaction, an increased PYK flux, an increased glycolytic flux, and a decreased PPP (which was optimally inactivated at the demarcation between regions 1 and 2). Regions 2 and 3 are very similar with respect to the set of metabolic reactions that are optimally utilized, and in these regions, the PPP is optimally inactivated. At the demarcation between regions 3 and 4, the maximal growth flux was about 95% of the *in silico* wildtype and the TCA cycle flux was increased to approximately 160% of the *in silico* wildtype. With TCA cycle flux increases beyond region 3, region 4 is encountered (Figure 7). Region 4 was characterized by alternate optimal flux distributions. The metabolic network was limited in the ability to produce the essential glycolytic and PPP biosynthetic precursors. In this region, the optimal growth flux was sensitive to the TCA cycle flux, and the metabolic fluxes in this region are not shown in Figure 8 because alternate optimal solutions exist.

Discussion

We have illustrated, with the complete *E. coli* metabolic network, how optimal metabolic phenotypes (flux vectors) and shifts in metabolic behavior can be analyzed and interpreted *in silico*. From the fundamental physicochemical constraints on the metabolic network, the feasible set that identifies the capabilities of the metabolic network was identified. Subsequently, a linear optimization routine was utilized to search the feasible set for a flux vector that maximizes a given objective function. Given the complexity associated with developing complete dynamic modeling of cellular processes, the constraining approach, as discussed herein, is a particularly useful alternative approach to metabolic systems analysis. The results presented herein are of fundamental interest for several reasons. First, the ability to define essential genes under various conditions will have many

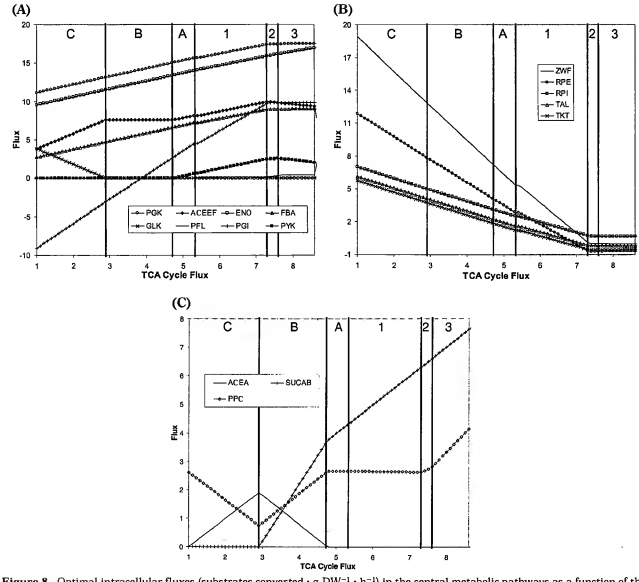

Figure 8. Optimal intracellular fluxes (substrates converted · g-DW^{-1} · h^{-1}) in the central metabolic pathways as a function of the TCA cycle metabolic flux constraint (substrates converted · g-DW^{-1} · h^{-1}). The glucose uptake rate was constrained to 10 mmol g-DW^{-1} h^{-1}. (A) Glycolytic fluxes. (B) Pentose phosphate pathway fluxes. (C) TCA cycle fluxes.

practical applications. Second, the results presented discussed the sensitivity of the objective function to specific fluxes in the metabolic network. Finally, the results demonstrate the potential capabilities of *in silico* analysis of cellular systems. Understanding the relation between individual fluxes and the holistic function of the metabolic network is essential to successfully metabolic engineering a living system, and FBA provides a methodology that can be used to direct the metabolic engineer.

First, we should address the assumptions associated with the utilization of linear optimization to identify the optimal flux vector. The metabolic constraining formalism that we have discussed is based on the fundamental physicochemical constraints that all cells must abide to. Within the set of constraints, the cell will choose a flux vector for which to operate. We have attempted to find the same flux vector by employing linear optimization, and the assumption is that the cell has evolved the regulatory mechanisms to find the optimal solution within the physicochemical constraints to maximize its survival. We have mathematically represented survival as cellular growth. On the basis of comparisons of the linear optimization results and experimental data, the assumption appears to be valid under the tested conditions (*27*). However, currently, the number of situations for which the validity of the assumption has been addressed is limited. Therefore, further experimental validation is in order. It should be noted that, even if the assumption of optimal growth proves correct for wildtype strains, it is not clear if we should expect that an engineered strain will behave in an optimal manner. Therefore, the optimization results may only provide an upper bound on the expected behavior of engineered strains.

The identification of the essential gene products in a metabolic network is of fundamental interest (*46, 47*). The metabolic constraining formalism that was described here provides an efficient method to study the consequences of alterations in the genotype and to gain insight into the genotype-phenotype relation. The study of the removal of individual metabolic enzymes in the central metabolic pathways demonstrated fundamental redundancy properties of the *E. coli* metabolic genotype and the existence of relatively few critical gene products. Seven metabolic reactions were determined to be ess-

ential in the metabolic network. Of these seven reactions, one set of three forms a linear reaction series and another set of two forms another linear reaction series; therefore, the effect of deleting any of the reactions in the linear set is equivalent. Thus, there were basically four different metabolic fluxes that were essential: two in the PPP, the 3-carbon stage of glycolysis, and the first three reactions of the TCA cycle. It was shown that there are regions where the ability of the metabolic network to support growth was not affected by the altered flux levels in the essential enzymatic reactions. This metabolic robustness was due to the capability of the metabolic network to shift the production of redox potential and high-energy phosphate bonds. However, the point where the metabolic network was unable to support growth was quantitatively derived *in silico*. At this point, the metabolic network was limited in the ability to produce biosynthetic precursors. In the biosynthetic precursor limited region, the availability of redox potential and high-energy phosphate bonds did not limit the growth capability of the cell.

The robustness analysis presented herein is essentially a sensitivity analysis. The sensitivity of the objective function to changes in the optimal flux vector was addressed. A constraint was added to the metabolic network, thus altering a single metabolic flux, and a new optimal flux vector was calculated. The relation between the additional constraint and the objective function was examined to investigate the robustness in the system with respect to the essential enzymatic reactions. Furthermore, the relation between the additional constraint and optimal flux vector was also examined. The results of the sensitivity analysis can be used to interpret the optimal metabolic fluxes and their relation to in vivo metabolic fluxes. For example, metabolic fluxes for which the objective function is highly sensitive are likely to obtain in vivo values that are consistent with the optimal values. Furthermore, metabolic fluxes to which the objective is not sensitive may be able to take on flux values in a large range with very little effect on the optimal solution. Additionally, other fluxes in the metabolic network that are sensitive to a given metabolic flux may provide an indication of the accuracy of the optimal predictions. For example, the TCA cycle flux was reduced to approximately 18% of the *in silico* wildtype with little effect on the objective function; however, the PPP fluxes were three times the *in silico* wildtype, fluxes much higher than experimental data indicates.

Understanding the metabolic fluxes and their control is essential to the ability to "design" metabolic networks for the production commodity chemicals (i.e., antibiotics, vitamins, amino acids, etc). Using flux balance analysis the complete range of metabolic phenotypes can be examined under defined environmental and genetic conditions through the use of phenotypic phase planes (PhPP). For the *E. coli* network PhPPs were generated for growth on glucose minimal media spanning the uptake rate of the carbon source and an intracellular metabolic flux. Therefore, the PhPP formalism has provided an efficient methodology for examining the consequence of altered fluxes within the cell. Therefore, bioinformatically based models will undoubtedly have a major impact on the development of metabolic engineering (*4*, *5*). Herein, we have investigated the effect of altered metabolic flux levels on the maximal growth flux, thus quantifying the relation between altered flux levels and optimal cellular growth. Furthermore, by examining the entire set of constraints on the metabolic network, constraints on metabolic flux alterations can be identified.

FBA incorporates no information on enzyme kinetics or gene regulation, thus limiting insight into dynamic responses. From flux balance analysis it is possible to realize some of the fundamental constraints that metabolic systems are faced with and define the feasible set that contains all admissible steady-state flux vectors. As in vivo reaction dynamics is further understood, the ability to predict dynamic responses of metabolic networks to environmental and genetic perturbations using dynamic modeling approaches will become more feasible. In general regulatory schemes and reaction dynamics will serve to further constrain metabolic behavior to operate in confined subspaces of the feasible set. Identifying these regions from both the theoretical and experimental side will be a challenge for the future.

A number of experimental technologies have now made the holistic study of biological systems feasible. The ability to assimilate DNA chip-based and protein expression technologies providing genome-scale information with computational methods for metabolic network analysis will become important in advancing the study of metabolic physiology and the practice of metabolic engineering. Currently the interpretation of high-throughput experimental information on systemic behavior is limited by a lack of analysis capabilities. Can systems-based quantitative *in silico* approaches such as flux balance be used to assist in understanding this flood of data? This question will need to be answered as interest builds in the genomics community for quantitative systems analysis.

The analysis of the metabolic phenotype–genotype relation using the bioinformatically based *in silico* metabolic genotype of *E. coli* can serve as a basis for the construction of parallel *in silico* representations of other single-cell organisms. Thus, the results presented are particularly relevant with the current emphasis on genome sequencing. Utilizing the techniques described herein, information can be gained regarding the metabolic physiology of a cell with relatively little experimental biochemical information on the cell of interest. However, this analysis should be considered a single step toward the integrative analysis of bioinformatic databases to predict and understand cellular function based on the underlying genetic content. Continued prediction and experimental verification will be an integral part of the further development of *in silico* strains and their use to represent their in vivo counterparts.

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The growing scope of applications of genome-scale metabolic reconstructions using *Escherichia coli*

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The number and scope of methods developed to interrogate and use metabolic network reconstructions has significantly expanded over the past 15 years. In particular, *Escherichia coli* metabolic network reconstruction has reached the genome scale and been utilized to address a broad spectrum of basic and practical applications in five main categories: metabolic engineering, model-directed discovery, interpretations of phenotypic screens, analysis of network properties and studies of evolutionary processes. Spurred on by these accomplishments, the field is expected to move forward and further broaden the scope and content of network reconstructions, develop new and novel *in silico* analysis tools, and expand in adaptation to uses of proximal and distal causation in biology. Taken together, these efforts will solidify a mechanistic genotype-phenotype relationship for microbial metabolism.

The availability of reconstructed metabolic networks for microorganisms has increased rapidly in recent years, and a growing number of research groups are reconstructing metabolic networks for organisms of interest[1]. A network reconstruction represents a highly curated set of primary biological information for a particular organism and thus can be considered a biochemically, genetically and genomically structured (BiGG) database[1,2]. A curated BiGG database (*de facto* a knowledge base) can be converted into a mathematical format (that is, an *in silico* model), and used to computationally assess phenotypic properties using a variety of computational methods[2,3]. Genome-scale reconstructions are thus a key step in quantifying the genotype-phenotype relationship and can be used to 'bring genomes to life'[4].

The purpose of this review is to summarize and classify applications using the *E. coli* reconstruction to answer a broad spectrum of biological questions. By doing so, we provide both an up-to-date overview of the applications of constraint-based analysis and a guide to similar applications for the growing number of organisms for which genome-scale reconstructions are becoming available.

Model formulation and the *E. coli* metabolic reconstruction

The four key steps in the formulation and use of genome-scale models are illustrated in **Figure 1**. Foundational to the process is the generation of global, or genome-scale, 'omics data'. Omics data, along with legacy information (that is, the 'bibliome') and small-scale detailed experiments, can be used to define the interactions among the biological components that are used to reconstruct organism-specific networks[1]. Network reconstruction is also an iterative, ongoing process that continually integrates data in a formal fashion as they become available[5]. As a result, a current and well-curated genome-scale network reconstruction is a common denominator for those studying systems biology of an organism; for an in-depth review of the bottom-up reconstruction process, see ref. 2.

The arrow from step 2 to step 3 in **Figure 1** involves a somewhat subtle, but critical, transition. With the definition of systems boundaries and other details, a network reconstruction can be converted into a mathematical format that can be computationally interrogated and subsequently used for experimental design[2]. Thus, a network reconstruction is converted into a genome-scale model (GEM)[3]. This arrow represents a bridge between the realms of high-throughput data/bioinformatics on the one hand and systems science on the other. A network reconstruction (or a BiGG knowledgebase) is, in principle, accessible to all and significant strides have been made to make computation with GEMs more readily accessible[6–11]. This availability of both genome-scale reconstruction and GEMs has unleashed creativity in research groups around the world and resulted in the series of studies reviewed below.

The 18-year history of reconstruction of the *E. coli* metabolic network (summarized in **Fig. 2**) has culminated in a network containing a total number of 1,260 open reading frame (ORF) metabolic functions[12–19]. This reconstruction represents 48% of the experimentally determined ORF functions in the *E. coli* genome (**Table 1**). It should be noted that the functions of 92% of the 1,260 gene products have been experimentally verified. Reconstruction of the *E. coli* network has thus approached an exhaustion of known metabolic gene functions and it is now being used in a prospective fashion to discover new metabolic capabilities (see below). The reconstruction of the *E. coli* metabolic network represents the best-developed genome-scale network to date and it has proven to be a platform for a variety of computational analyses. It should be noted that although there are different *E. coli* GEMs, there is only one unique network for *E. coli* and each successive iteration strives to best represent this content (we use 'the *E. coli* GEM' to refer to any of these iterations). Three successive *E. coli* GEMs from our group[17–19] have been used as the basis for over 60 detailed studies reviewed below.

A growing number of research groups use the *E. coli* GEM for predicting, interpreting and understanding *E. coli* phenotypic states and function.

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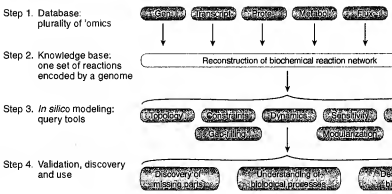

Figure 1 Formulation and use of GEMs as a four-step process. Step 1, the process is based on a variety of high-throughput data sets (that is, omics data) and a comprehensive assessment of the literature (that is, bibliomic data). Step 2, all of the data types are used to reconstruct the list of biochemical transformations that make up a network, as well as their genetic basis[1]. In principle, the network is unique. Step 3, the data contained in the reconstruction can be formally represented (that is, in the form of matrices and logical statements) that can be mathematically characterized by a variety of methods. Step 4, the computational model enables a broad spectrum of applications, as reviewed in this article. Figure adapted from ref. 2.

In addition, the reconstruction itself has been used as a context for the interpretation of large amounts of experimental data. Applications of the *E. coli* GEM range from pragmatic to theoretical studies, and can be classified into five general categories (**Fig. 3**): first, metabolic engineering[20–30]; second, biological discovery[31–37]; third, assessment of phenotypic behavior[19,38–63]; fourth, biological network analysis[64–79]; and fifth, studies of bacterial evolution[80–82]. The *in silico* methods used to probe the *E. coli* GEM in each study are summarized in **Figure 4**. These methods perform an assessment of the solution spaces associated with the mathematical representation of a reconstruction[2]; they are categorized as either unbiased or biased[3]. The latter category relies on an observer bias that is stated through an objective function (that is now beginning to be experimentally examined[83]) that has been utilized in most of the studies reviewed here for the general application of flux balance analysis (FBA)[84–86]. Each category of application is now detailed, with emphasis on the first three that have the greatest practical utility.

GEMs and metabolic engineering

Through the application of computational methods that incorporate linear, mixed integer linear and nonlinear programming, it has been demonstrated that model-directed strain design can lead to increased metabolite production[20–30]. In these studies, the *E. coli* GEM is principally used to analyze the metabolite production potential of *E. coli* and identify metabolic interventions needed to produce the metabolite of interest. Thus, *E. coli* strains have been systematically designed through *in silico* analysis to overproduce target metabolites, such as lycopene[23,24], lactic acid (our group[25]), ethanol[26], succinic acid[27,28], L-valine[29], L-threonine[30], additional amino acids[21] as well as diverse products from hydrogen to vanillin[22].

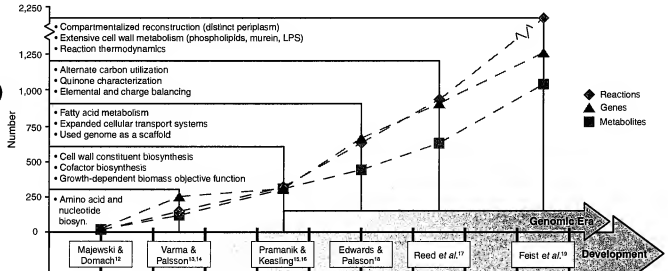

Figure 2 The iterative reconstruction and history of the *E. coli* metabolic network. Six milestone efforts are shown that contributed to the reconstruction of the *E. coli* metabolic network. For each of the six reconstructions[12–19], the number of included reactions (blue diamonds), genes (green triangles) and metabolites (purple squares) are displayed. Also listed are noteworthy expansions that each successive reconstruction provided over previous efforts. For example, Varma & Palsson[13,14] included amino acid and nucleotide biosynthesis pathways in addition to the content that Majewski & Domach[12] characterized. The start of the genomic era[92] (1997) marked a significant increase in included reconstruction components for each successive iteration. The reaction, gene and metabolite values for pregenomic-era reconstructions were estimated from the content outlined in each publication and in some cases, encoding genes for reactions were unclear.

Select exemplary metabolic engineering applications are described in more detail below.

To increase the production of an already high producing strain, a systematic computational search was developed[24] to explore the *E. coli* metabolic network and report gene deletions that diverted metabolic flux toward the desired product lycopene. This process resulted in a knockout strain, that when constructed, showed a twofold increase in the production of lycopene over the parental strain. In this analysis, the computational algorithm MOMA (minimization of metabolic adjustment)[41] and the *i*JE660 (ref. 18) *E. coli* GEM were used to sequentially examine additive genetic deletions that would improve lycopene production while maintaining cell viability. Strain designs were constructed through genetic manipulations using the predicted modifications and this computational approach yielded a twofold increase in production rate over a previously engineered overproducing strain and an 8.5-fold increase over wild-type production harboring only a lycopene biosynthesis plasmid[24]. Strain performance was evaluated by monitoring lycopene production through enzymatic assays and mutant growth rates. In addition, when the strain designs identified computationally were compared with mixed combinatorial transposon mutagenesis, the maximum observed production could be designed solely using the systematic GEM-aided computational method[23,24]. Furthermore, a deleterious effect was observed when targets identified in individual computational designs were combined in an attempt to achieve an overall more desirable phenotype. Thus, the overall systematic effects from individual designs were not additive and needed to be interpreted in the context of the entire network.

Table 1 Properties of the most current *E. coli* metabolic reconstruction[19]

Included genes	1,260	(28%)[a]
Experimentally based function	1,161	(92%)
Computationally predicted function	99	(8%)
Unique functional proteins	1,148	
Multigene complexes	167	
Genes involved in complexes	415	
Instances of isozymes[b]	346	
Reactions	2,077	
Metabolic reactions	1,387	
Unique metabolic reactions[c]	1,339	
Cytoplasmic	1,187	
Periplasmic	192	
Extracellular	8	
Transport reactions	690	
Cytoplasm to periplasm	390	
Periplasm to extracellular	298	
Cytoplasm to extracellular	2	
Gene–protein reaction associations		
Gene associated (metabolic/transport)	1,294/625	
Spontaneous/diffusion reactions[d]	16/9	
Total (gene associated and no association needed)	1,310/634	(94%)
No gene association (metabolic/transport)	77/56	(6%)
Exchange reactions	304	
Metabolites		
Unique metabolites[c]	1,039	
Cytoplasmic	951	
Periplasm	418	
Extracellular	299	

[a]Overall genome coverage based on 4,453 total ORFs in *E. coli* iAF1260 contains 48% of the ORFs in *E. coli* that have been characterized experimentally (2403 ORFs)[99]. [b]Tabulated on a reaction basis, not counting outer membrane nonspecific porin transport. [c]Reactions can occur in or between multiple compartments and metabolites can be present in more than one compartment; [d]Diffusion reactions do not include facilitated diffusion reactions and are not included in this total if they can also be catalyzed by a gene product at a higher rate.

Two studies producing the amino acids L-valine[29] and L-threonine[30] have demonstrated the broad usage of GEM-aided computation for strain design. To generate an L-threonine production strain, GEM-aided modeling was employed in three different areas to increase production to industrial titers[30]. In one instance, *in silico* parametric sensitivity analysis that compared reaction activity to L-threonine production rate was used to identify the optimal activity of a key enzymatic reaction associated with maximal L-threonine production. The optimal activity prediction was subsequently used to tune the overexpression of the gene encoding the enzyme involved in this reaction through comparison to base-line activity and the result was a production increase. This method proved to be vital to the success of this strain, as a previous transcription-profiling guided attempt at overexpression resulted in an undesirable surplus of activity and was detrimental to L-threonine production. For the same strain, a GEM-aided flux analysis in conjunction with mRNA expression data levels also was used as a guide for elimination of negative regulation of a gene encoding an enzyme involved in a reaction that channeled flux toward the final product. The third use of the GEM for the design of this strain occurred when an unwanted byproduct was observed in the culture medium and computation was used to divert the flux from this byproduct to L-threonine[30] through overexpression of another key gene-encoded activity.

The second analysis applied the systematic computational search algorithm previously described[24] to the updated *E. coli* GEM MBEL979 (ref. 7) (which is similar to the *i*JR904 GEM[17]) to improve L-valine production. The *in silico* analysis of beneficial knockouts to divert flux toward the desired product once again resulted in a significant increase in the production of the desired metabolite over that of an existing overproducing strain, in this case, a more than a twofold increase[29]. Furthermore, the authors followed several additional metabolic engineering approaches to increase overproduction (that is, relieving feedback inhibition and regulation through attenuation, removing competing pathways, upregulation of primary biosynthetic pathways and overexpression of exporting machinery). When compared with each of the other individual strain modifications, the *in silico* GEM-aided interventions resulted in the greatest increase in L-valine production[29]. Taken together, these two studies demonstrate the broad applications for which GEMs can be used not only to design strains in a *de novo* fashion, but also to make further improvements on strains through integrating and interpreting experimental data.

Several other strain designs using *E. coli* GEMs have been reported. In a combined computational and experimental study, our group[25] has used the bi-level optimization algorithm OptKnock[20] and *i*JR904 (ref. 17) to overproduce lactate in *E. coli*. The algorithm OptKnock optimizes two objective functions, biomass formation and product secretion, to assist in the creation of strains that can couple the excretion of a desirable product

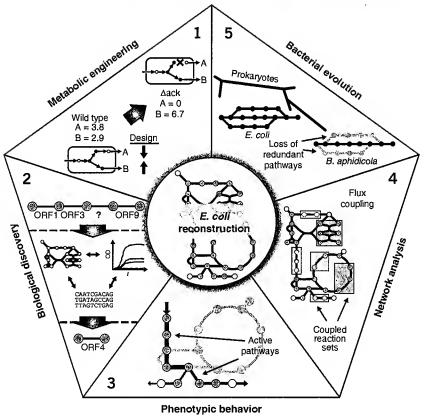

Figure 3 Applications of the genome-scale model (GEM) of *E. coli* divided into five categories. (1) A drawing of a predicted effect from a loss-of-function mutation in a simple system is shown. Metabolic engineering studies have investigated *in silico* strain design using *E. coli* metabolic reconstructions to overproduce desired products[20–30]. (2) Recent studies using the reconstruction in a prospective manner have aimed to use the current biochemical and genetic information included in the metabolic network along with additional data types to drive biological discovery, such as predicting genes responsible for orphan reactions[32,33,35–37]. (3) Using the reconstruction in phenotypic studies, computational analyses have examined gene[19,46,51,53,63], metabolite[44,60] and reaction[39,47,48,58] essentiality along with considering thermodynamics[19,40,47,49,52,54,55,57,61] to make better predictions about the physiological state (that is, the active pathways) of the cell for a given environmental condition. (4) The *E. coli* reconstructions have been used to analyze and interpret the intrinsic properties of biological networks, one example being finding coupled reaction activities[66] (as shown in the drawing) across different growth conditions. (5) Using the network reconstruction, evolutionary studies have examined the cellular network in the context of adaptive evolution events[81], horizontal gene transfer[80,81] and minimal metabolic network evolution[82].

to the growth rate. Using adaptive evolution with growth-rate selection pressure, we found that the lactate-producing strains designed using OptKnock possess this growth-coupling property (as measured by growth rate, uptake and secretion rate profiles); thus, this study demonstrated the utility of adaptive evolution as a design tool[87].

Additional noteworthy examples of GEM-aided design are two studies[27,28] demonstrating the use of GEM modeling based on iJR904 (ref. 17) in screening for genes of putative importance in succinate production. Combinatorial knockouts that were predicted to be overproducers *in silico* were experimentally verified to display the same overproducing phenotype *in vivo*. Furthermore, this method was shown to be superior to the use of comparative genomics for strain design, which was also performed in one of the studies[27].

A growing number of metabolic engineering studies thus demonstrate the use of GEMs to generate strain designs that are often nonintuitive and nonobvious. An excellent example of a nonintuitive strain improvement outlined in this section was when modeling was used not only to study the effect of a gene removal, but also to tune the expression of a gene to an optimally predicted level, which when surpassed, was detrimental to product formation. In this manner, genome-scale reconstructions allow the examination and simulation of metabolism as an integrated network, circumventing the possible shortcomings of methods that rely on manual

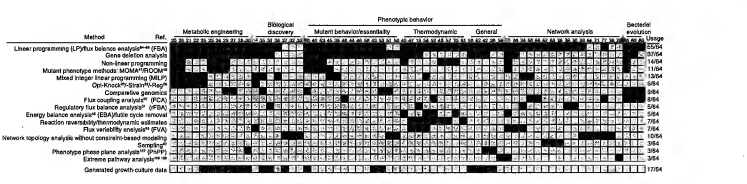

Figure 4 Summary of the *in silico* methods used in the 64 published *E. coli* GEM studies reviewed here. This heat map characterizes the incorporation of different computational methods into studies using genome-scale models of *E. coli*[20,22,25,30,40,41,45,65–67,84–85,106–108]. A dark box indicates that a particular method (one method per row) was used in a corresponding study (one citation per column); the frequency of usage of a particular method is given on the right. Studies were grouped into one of five general categories, and studies examining phenotypic behavior were further divided into three subgroups. Studies that contributed new experimental growth data are also marked along the bottom offset row.

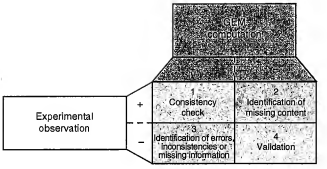

Figure 5 Comparison of computation and experimental data: identification of agreements and disagreements. The comparison of GEM computation and organism-specific experimental measurements identifies agreements and disagreements. For comparison, phenotypic outcomes are tabulated for genetic perturbations examined in a given environment (e.g., growth or no growth) for both experiment and computation. A '+' indicates that a given phenotype is not affected by the perturbation; an '–' indicates it does. Each outcome of comparison has a different implication: 1, consistency check, a perturbation has no affect on the property being measured and modeling predicts the same; 4, validation, the perturbation affects the experimental outcome and modeling with the GEM predicts this outcome; 2, identification of missing content—when GEM modeling fails to predict the positive confirmation of the property being measured, this outcome indicates that there is missing content in the GEM and can lead to the identification of specific areas for biological discovery; 3, identification of errors, inconsistencies or missing context-specific information—a positive prediction for the measured property and an opposite experimental observation indicates a possible error in the current organism-specific knowledge or that additional context-specific information is lacking from the GEM or modeling method (e.g., transcriptional regulation).

assessment of a limited number of interactions and fail to detect non-intuitive causal interactions. With the growing availability of organism- and strain-specific GEMs, applications for designing microbial strains for industrial production are expected to continue to grow. This growth expectation is in part based on the ongoing reconstruction of additional cellular processes, such as transcriptional regulation and protein production. Computations based on genome-scale models are also beginning to influence other areas of industrial microbiology such as generation of renewable energy[88–90] and bioremediation[89].

GEM-driven discovery in *E. coli*

GEMs can also provide a guide to biological discovery. This capability is based on comparison of computed and actual experimental outcomes. Given the fact that BiGG knowledge bases are incomplete and that they contain gaps[91], they provide a context for systematic discovery of missing information. The comparison between computation and experiments is summarized in **Figure 5** highlighting how agreements and disagreements are analyzed.

The current area of most interest is direct discovery efforts toward characterizing unknown ORFs in the *E. coli* genome. Ten years after the first release of the complete genome sequence[92], many unknown ORFs still exist in the *E. coli* genome (**Supplementary Table 1** online), with many of these likely to encode metabolic functions. ORF discovery using GEMs also has significant potential to affect not only how new and less studied genomes are annotated, but to fill out the missing pieces of *E. coli* metabolism.

To address this challenge, researchers have developed algorithms to determine the probable gene candidates that fill knowledge gaps in the *E. coli* and other network reconstructions. These algorithms use global network topology and genomic correlations, such as genome context and protein fusion events[32], as well as local network topology and/or phylogenetic profiles[32,33]. Similar tools have been developed that use mRNA coexpression[93] and which can evaluate more general metabolic pathway databases[94]. In addition to these network topology-based methods, an optimization-based procedure has also been developed to fill network gaps and evaluate reaction reversibility along with adding additional transport and intracellular reactions from databases of known metabolic reactions[36]. These studies produce specific targets for drill-down (that is, detailed biochemical characterization) experiments needed for confirmation of these computationally generated hypotheses.

Two recent studies have integrated a combined computational and experimental approach to aid the ORF discovery process in *E. coli* through using a GEM and high-throughput phenotype data[35,37]. The first study (from our group[35]) used an iterative process in which (i) differences in modeling predictions and high-throughput growth phenotype data were identified, (ii) potential missing reactions that remedy these disagreements were algorithmically determined, (iii) bioinformatics was used to identify likely encoding ORFs and (iv) resulting targeted ORFs were cloned and experimentally characterized. Application of this process led to the functional characterization of eight ORFs involved in transport, regulatory and metabolic functions in *E. coli*[35]. The discovery process was aided by a high-throughput growth phenotyping analysis and the genome-wide single-gene mutant collection[95], along with other characterization analyses such as targeted expression profiling.

The second GEM-based analysis resulting in ORF discovery used network topology to examine orphan reactions in the *E. coli* network (that is, reactions known to exist in *E. coli* that have not been linked to an encoding gene) identified by the previously mentioned network topology-based gap-filling algorithms[32,33,93]. The basic premise behind these algorithms is the utilization of an orphan reaction's network neighbors as constraints to assign metabolic function. With the resulting tentative ORF assignment, biochemical characterization studies using genetic mutants[95], analysis of growth under different substrate conditions and expression data were all used to characterize and assign function to an orphan ORF that is responsible for a metabolic conversion that has been known for 25 years[37].

Further studies in this category of biological discovery applications (not focused on ORF identification) have used GEMs of *E. coli* to identify potential bottleneck reactions in the metabolic network[34] and as of yet uncharacterized transcription factor target interactions in *E. coli*[31]. The former study, targeting the elucidation of regulatory and metabolic interactions in *E. coli*, developed an iterative procedure focused on reconciling computational and experimental discrepancies stemming from high-throughput growth phenotype and gene expression data where selected expression changes were validated using RT-PCR[31]. With the advancement of high-throughput technologies to test the hypotheses generated from computational studies, these and similar algorithmic approaches are likely to continue to aid in the quest to achieve full functional annotation of the *E. coli* genome and its context-specific uses.

GEM-aided phenotypic assessment

The area where the *E. coli* GEMs has been most extensively used is for the examination and quantitative interpretation of metabolic physiology for wild-type, genetically perturbed and adaptively evolved strains of *E. coli*[19,38–63]. These efforts have implications in both the quantitative and

qualitative understanding of physiological states of the cell. Furthermore, these efforts have examined *E. coli* physiology for a vast number of given genetic and environmental conditions and incorporation of the developed methods will have an impact on future design of biological systems and modeling approaches. A large subset of these studies of phenotypic behavior aim to use thermodynamic laws and information to refine phenotype predictions of GEMs and to incorporate metabolomic and fluxomic data into modeling[19,40,47,49,52,54,55,57,61].

A set of distinct computational methods using GEMs has been developed to determine the physiological state of *E. coli* after genetic perturbations[41,45,50]. These studies have used ^{13}C flux measurements and growth-rate phenotype data to evaluate the predictability of the developed algorithms when compared to experimental observations. Whereas comparisons to flux data from wild-type and *E. coli* mutants reveal that the computational algorithm MOMA[41] provides better predictions for transient growth rates (early post-perturbation state), the algorithm ROOM (Regulatory on/off minimization)[45] and basic FBA was more successful in predicting final steady-state growth rates and overall lethality[45]. These algorithms have been used, in addition to basic FBA, for genome-wide essentiality screens, as outlined below.

A range of computational studies have sought to understand phenotypes through determining the essential genes[19,46,51,53,63], metabolites[44,60] and reactions[39,47,48,58] in the *E. coli* metabolic network. A common benchmark for examining GEM predictive ability is to determine the agreement with growth phenotype data from knockout collections of *E. coli*. Such studies (e.g., refs. 19,53) will be further enabled by a comprehensive single-gene knockout library for *E. coli* recently made available[95]. Implications for examining network essentiality in *E. coli* include determining network essentiality in similar organisms[39,48,53,58], deciphering network makeup and enzyme dispensability (that is, measures of robustness)[46,58,60], aiding in metabolic network annotation, validation and refinement[44], and even rescuing knockout strains through additional gene deletions[63], to name a few. The predictive capability of the *E. coli* GEM, as demonstrated by these studies, has been instrumental in adapting it for different uses. One particular study examining knockout phenotypes has demonstrated that the *E. coli* GEM was able to predict the outcomes of adaptively evolved strains to a high degree (78%) when knockout *E. coli* strains were grown in several different substrate environments by examining growth rates at the beginning and end of adaptive evolution[43]. This study represents a demonstration of a GEM's ability to look at adaptive behavior (or 'distal' causation[96]), in addition to immediate behavior (or 'proximal' causation[96]). Predictive capability is expected to improve through examining growth behavior across a greater number of environments (additional phenotyping screens will be necessary) and with an increase of integration of additional cellular processes[31]. Genetic perturbations have played a key role in the study of the genotype-phenotype relationship in biology and GEMs can be used to mechanistically interpret the results and predict the outcomes of such perturbations.

Incorporating thermodynamic information into *E. coli* GEMs has shown promise in narrowing predictions of allowable physiological states in a given environment[19,40,47,49,52,54,55,57,61] and in identifying reactions likely to be subject to active allosteric or genetic regulation[49,54]. This field is progressing rapidly and should prove to increase the predictive capabilities of genome-scale modeling through the addition of governing thermodynamic physiochemical constraints. One particular analysis incorporating compound formation and reaction energies for the content of the GEM based on iJR904 (ref. 17) identified reactions that are likely to be effectively irreversible for any realistic metabolite concentration[54]. The hypothesis was advanced that these reactions are candidates for cellular regulation in their respective pathways because enzyme regulation will likely be the dominant mechanism for control of flux through these reactions[54].

The addition of thermodynamics in GEM modeling enables the analysis of metabolomic data in the context of a reconstruction. A study using high-throughput metabolomic data and GEMs proposed likely regulatory interactions by deciphering the metabolite concentrations in the context of overall network functionality[49]. Not only did the metabolomic data benefit computations by constraining the system using physiological measurements, but the computational predictions were also able to validate quantitative metabolomic data sets for consistency through providing a functional context to relate metabolite concentrations. This application is one example of how metabolomic data will directly influence modeling and how metabolite concentration data are likely to greatly influence future metabolic modeling owing to its intimate connection with GEM content. Similar work incorporating other quantitative values with FBA, such as metabolite concentrations[57] and flux ratios at branch points in metabolism[56], is also appearing.

Applying a different physiochemical constraint, molecular crowding, a framework has also been developed to incorporate spatial constraints into FBA[59]. The functional states predicted with this method (that is, FBA with molecular crowding; FBAwMC) and the *E. coli* GEM were validated against generated growth, substrate and production rate data along with gene expression profiles and enzyme activity measures to demonstrate predictive accuracy, including substrate preferentiality, when examining growth in complex substrate environments[59,62]. Overall, these studies, which incorporate reaction thermodynamics and additional cellular constraints, should further narrow the range of allowable functional network states that can made based on stoichiometry alone and thus improve the utility of GEMs.

In addition to analyses on the genomic scale, several studies modeling the metabolism of *E. coli* on a smaller scale have been performed. These analyses typically use models containing ~100 reactions or less and most often focus on incorporating nonlinear analysis to understand quantitative experimental data (e.g., isotopomer modeling). With the advancement of computational power and developed platforms, the networks that can be analyzed will grow in size[97]. Given that the results produced from such analyses as isotopomer modeling have been shown to be highly dependent on the content of a reduced model, the logical starting point for building such models is the *E. coli* GEM[97]. Several noteworthy studies have been conducted with reduced models, but are not detailed here as they are outside the scope of this review.

GEMs and network property analysis

E. coli is generally viewed as having the most complete characterization of any model organism[98,99]. Because of the incorporation of thousands of metabolic interactions with relatively high reliability (e.g., 92% of the genes included in the latest reconstruction of *E. coli*[19] have experimentally determined annotated functions[98]; **Table 1**), validated genome-scale reconstructions of *E. coli* have become popular resources for the analysis of various network properties[64–79]. The methods designed to analyze the underlying network structure of *E. coli* metabolism, some characterizing its interplay with regulation, have been developed to determine several physiological features. These features include the most probable active pathways and metabolites used under all possible growth conditions[67,69,73,75], the existence of alternative optimal solutions and their physiological significance[65], conserved intracellular pools of metabolites[68], coupled reaction activities[66] and their relationship to gene co-expression[77], metabolite coupling (our laboratory[71]), metabolite utilization[72], the organization of metabolic networks[64,76], strategies for *E. coli* to incorporate metabolic redundancy[78] and the dominant functional states of the *E. coli* network across various environments[70,74,79]. These findings are driven both by biased approaches using FBA and biomass objective function optimization and by unbiased approaches

such as graph-based analyses (see Fig. 4). One noteworthy study using the GEM-outlined network examined thousands of different potential growth conditions and observed a 'high-flux backbone' in *E. coli* that both carried high levels of flux across the different environmental conditions and was composed of a relatively small set of enzymatic reactions[67]. This result can be of practical importance for synthetic biology efforts aimed toward manipulating flux within biological systems. Furthermore, this finding was hypothesized to be a universal feature of metabolic activity in all cells and was consistent with flux measurements from ^{13}C-labeling experiments[67].

The studies in this category have a common systems biology theme; namely, the development and subsequent demonstration of methods that identify sets of reactions or metabolites with correlated or coordinated functions and systematic relationships. The systems biology that these methods enable and demonstrate has potential implications for (i) antimicrobial drug-target discovery[68,69], (ii) aiding the development of additional metabolic reconstructions[66,68], (iii) guiding genetic manipulations[66], (iv) improving metabolic engineering applications[67,68] and (v) increasing the general understanding of biological network behavior[65,74,77] and resilience[78]. The role that the *E. coli* GEM has taken is a comprehensive and curated set of up-to-date metabolic knowledge; thus providing a scaffold for these large-scale computations.

GEMs and bacterial evolution

The GEMs of *E. coli* metabolism have been used to examine the process of bacterial evolution[80–82]. Specifically, the network reconstructions have been used to interpret adaptive evolution events[81], horizontal gene transfer[80,81] and evolution to minimal metabolic networks[82]. These studies, which use the *E. coli* reconstruction as an organism-specific genetic and metabolic content database, and the corresponding GEM, have been able to provide insight into evolutionary events through combining known physiological data (e.g., in various environmental conditions) with hypotheses and *in silico* computation. Examining the evolution of minimal metabolic networks through simulation demonstrated that it was possible to predict the gene content of close relatives of *E. coli* by examining the necessity of genes and reactions in the overall context of the system functionality for a specific lifestyle[82]. Similarly, by re-examining network functionality in a number of different environments and through the use of comparative genomics, it has been shown that recent evolutionary events (e.g., horizontal gene transfer) probably resulted from a response to a change in environment[81]. Furthermore, computational analysis led to the additional conclusion that these horizontal gene transfer events are more likely if the host organism contains an enzyme that catalyzes a coupled metabolic flux related to the transferred enzyme's function[80,81]. Taken together, these studies demonstrate the importance of having high-quality curated reconstructions to enable studies on an organism's response to environmental changes and for understanding the fundamental forces driving bacterial evolution.

Conclusions

Since the first review on constraint-based methods appeared in *Nature Biotechnology* in 1994 (ref. 84), the field has grown rapidly. The myriad studies described in this article highlight the rapid development and use of genome-scale reconstruction and derived computational models to address a growing spectrum of basic research and applied problems. Experience with genome-scale reconstructions has demonstrated that they are a common denominator in the systems analysis of metabolic functions. With the recognition of its basic paradigms and a growing spectrum of practical uses enabled, this field now faces several exciting challenges in three major areas, including: first, network reconstructions and the reconstruction process; second, computational BiGG query tools (that is, modeling); and third, application to proximal and distal causation in biology.

The scope of reconstructions is bound to grow, representing more and more BiGG knowledge in the structured format of a GEM[91]. Growth in scope in the near term will on one front involve the transcriptional and translational machinery of bacterial cells[100–102]. Such an extension will enable a range of studies including the direct inclusion of proteomic data, fine graining of growth requirements and the explicit consideration of secreted protein products. Another expansion in scope in the near term is the reconstruction of the genome-scale transcriptional regulatory network (TRN). Such reconstruction at the genome-scale is now enabled by new experimental technologies, such as chromatin immunoprecipitation (ChIP)-chip[103]. Experimental interrogation of the currently available TRN suggests that we know about one-fourth to one-third of its content[31], indicating that there is much to be discovered. Once reconstructed, the TRN will allow computational predictions of the context-specific uses of the *E. coli* genome and the responses of two-component signaling systems. Taken together, these near-term expansions in content will encompass the activity of apparently 2,000 ORFs in the *E. coli* genome.

Mid-term expansions in scope will include the growth cycle, shock responses and additional cellular functions. Such a reconstruction should eventually be a comprehensive representation of the chemical reactions and transactions enabled by *E. coli*'s gene products. Longer-term reconstruction may begin to address the three-dimensional organization of the bacterial cell. In particular, high-resolution ChIP-chip data on DNA-binding proteins could enable not only the estimation of the topological arrangement of the genome but also the elucidation of the structure of the cell wall and other cellular structures that will allow us a full three-dimensional reconstruction of *E. coli*.

We now know how to represent BiGG data in either a stoichiometric format or in the form of causal relationships (e.g., see ref. 104 from our laboratory) and how to use them to perform several lines of computational inquiries. Computational query tools of GEMs will continue to be developed. New advances will likely include modularization methods, use of fluxomic data and eventually kinetics. As the scope and content of the reconstruction grows, the need to modularize its content becomes more pressing. Fine- or coarse-grained views of cellular processes are needed for different applications. For instance, as previously mentioned, current computational limitations force the reduction in a network for the analysis of isotopomer data, and a rational way to carry out such reduction is needed. Given the systemic nature of fluxomic data and its phenotypic relevance, there is a pressing need to increase the size of the networks that can be analyzed for experimental measurement and estimation of flux states. Finally, although detailed kinetic models of microbial functions may currently be mostly of academic interest, we will most likely be able to construct them in the mid-term based on advances with metabolomic and fluxomic data, in addition to the developments that are occurring with the incorporation of thermodynamic information. Such large-scale kinetic models are likely to differ from those resulting from traditional approaches for construction of kinetic models as they come with different challenges.

As this article shows, the scope of applications of genome-scale reconstructions and GEMs is growing. Going forward, we wish to comment on three categories of applications: growth in coverage (that is, gap-filling), engineering (that is, synthetic biology) and the development of our understanding of fundamental biology (see Step 4, **Fig. 1**). Growth in coverage will come through discovery of missing network components. For instance, the latest metabolic reconstruction, iAF1260, contains 14% blocked reactions[19]. This disconnected content means that we have knowledge gaps that have arisen due to characterization of individual gene products outside the context of a given physiological function (that

is, outside a defined pathway). Metabolomic profiling is one measure that will provide us with the missing upstream or downstream routes to such dead ends in the network. Also, an expansion of scope in modeling will allow further investigation of network content, such as tRNA-charging reactions that are currently in this blocked reaction set[19]. Furthermore, growing metabolomic data suggest that we are discovering the existence of several new metabolites. Pathways that include these metabolites need to be discovered. Methods exist to compute missing pathways between molecules[105] that can be applied to such data. Such pathways, in turn, will lead to experimental programs to discover novel gene functions and to validate or refute the existence of such pathways. Similarly, we expect that a number of the components of TRNs are missing, such as new small RNA molecules (see **Supplementary Table 1**). Clearly, maintaining the quality control/quality assurance of such reconstructions will help in guiding us to a comprehensive genome-scale representation of all major cellular processes in bacteria at the BiGG data level of resolution that, in turn, enables GEMs of growing coverage and resolution.

Predictive models allow design. In fact, in engineering, there is 'nothing more useful than a good theory'. As this article demonstrates, genomics and high-throughput technologies have enabled the construction of predictive computational models. The scope of such predictions is limited at the moment, but with the growing scope and coverage of genome-scale reconstructions and advancements in the development of computational tools, this scope will broaden. Not only will GEMs influence design in synthetic biology, but their influence in discovery of cellular content will provide a more complete picture of the environment (that is, the parts list in the cell) in which future synthetically engineered constructs and circuits will be placed. The impact of GEMs on synthetic biology is thus likely to be notable; ranging from the provision of the cellular context of a small-scale gene circuit design to engineering of the entire genome-scale network toward fundamentally new and useful (that is, production) phenotypes.

Finally, we can speculate about the deep scientific impact that comprehensive predictive GEMs will have on our understanding of the living process. A comprehensive view of cellular functions will allow us to study the fundamental properties of both the underlying energy and information flows in living organisms. Such a view is likely to deeply affect our understanding of both distal and proximal causation in biology.

Note: Supplementary information is available on the Nature Biotechnology website.

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The *Escherichia coli* MG1655 *in silico* metabolic genotype: Its definition, characteristics, and capabilities

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The *Escherichia coli* MG1655 genome has been completely sequenced. The annotated sequence, biochemical information, and other information were used to reconstruct the *E. coli* metabolic map. The stoichiometric coefficients for each metabolic enzyme in the *E. coli* metabolic map were assembled to construct a genome-specific stoichiometric matrix. The *E. coli* stoichiometric matrix was used to define the system's characteristics and the capabilities of *E. coli* metabolism. The effects of gene deletions in the central metabolic pathways on the ability of the *in silico* metabolic network to support growth were assessed, and the *in silico* predictions were compared with experimental observations. It was shown that based on stoichiometric and capacity constraints the *in silico* analysis was able to qualitatively predict the growth potential of mutant strains in 86% of the cases examined. Herein, it is demonstrated that the synthesis of *in silico* metabolic genotypes based on genomic, biochemical, and strain-specific information is possible, and that systems analysis methods are available to analyze and interpret the metabolic phenotype.

bioinformatics | metabolism | genotype-phenotype relation | flux balance analysis

The complete genome sequence for a number of microorganisms has been established (The Institute for Genomic Research at www.tigr.org). The genome sequencing efforts and the subsequent bioinformatic analyses have defined the molecular "parts catalogue" for a number of living organisms. However, it is evident that cellular functions are multigeneic in nature, thus one must go beyond a molecular parts catalogue to elucidate integrated cellular functions based on the molecular cellular components (1). Therefore, to analyze the properties and the behavior of complex cellular networks, one needs to use methods that focus on the systemic properties of the network. Approaches to analyze, interpret, and ultimately predict cellular behavior based on genomic and biochemical data likely will involve bioinformatics and computational biology and form the basis for subsequent bioengineering analysis.

In moving toward the goal of developing an integrated description of cellular processes, it should be recognized that there exists a history of studying the systemic properties of metabolic networks (2) and many mathematical methods have been developed to carry out such studies. These methods include approaches such as metabolic control analysis (3, 4), flux balance analysis (FBA) (5–7), metabolic pathway analysis (8–11, 69), cybernetic modeling (12), biochemical systems theory (13), temporal decomposition (14), and so on. Although many mathematical methods and approaches have been developed, there are few comprehensive metabolic systems for which detailed kinetic information is available and where such detailed analysis can be carried out (see refs. 15–17 for a few noteworthy exceptions).

To analyze, interpret, and predict cellular behavior, each individual step in a biochemical network must be described, normally with a rate equation that requires a number of kinetic constants. Unfortunately, it currently is not possible to formulate this level of description of cellular processes on a genome scale. The kinetic parameters cannot be estimated from the genome sequence and these parameters are not available in the literature. In the absence of kinetic information, it is, however, still possible to assess the theoretical capabilities of one integrated cellular process, namely metabolism, and examine the feasible metabolic flux distributions under a steady-state assumption. The steady-state analysis is based on the constraints imposed on the metabolic network by the stoichiometry of the metabolic reactions, which basically represent mass balance constraints. The steady-state analysis of metabolic networks based on the mass balance constraints is known as FBA (7, 18, 19). This analysis differs from detailed kinetic modeling of cellular processes, in that it does not attempt to predict the exact behavior of metabolic networks. Rather it uses known constraints on the integrated function of multiple enzymes to separate the states that a system can reach from those that it cannot. Then within the domain of allowable behavior one can study the genotype-phenotype relation, such as the stoichiometric optimal growth performance in a defined environment.

In this manuscript, we have used the biochemical literature, the annotated genome sequence data, and strain-specific information, to formulate an organism scale *in silico* representation of the *Escherichia coli* MG1655 metabolic capabilities. FBA then was used to assess metabolic capabilities subject to these constraints leading to qualitative predictions of growth performance.

Materials and Methods

Definition of the *E. coli* MG1655 Metabolic Map. An *in silico* representation of *E. coli* metabolism has been constructed. We have used the biochemical literature (20), genomic information (21), and the metabolic databases (22–24). Because of the long history of *E. coli* research, there was biochemical or genetic evidence for every metabolic reactions included in the *in silico* representation, and in most cases, there was both genetic and biochemical evidence (Table 1). The complete list of genes included in the *in silico* analysis is shown in Table 1, and the metabolic reactions catalyzed by these genes can be found on the web (http://gcrg.ucsd.edu/downloads.html). The stoichiometric coefficients for each metabolic reaction within this list were used to form the stoichiometric matrix S.

Determining the Capabilities of the *E. coli* Metabolic Network. The theoretical metabolic capabilities of *E. coli* were assessed by FBA

Abbreviations: FBA, flux balance analysis; LP, linear programming; TCA, tricarboxylic acid; PPP, pentose phosphate pathway.

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Table 1. The genes included in the *E. coli* metabolic genotype (21)

Central metabolism (EMP, PPP, TCA cycle, electron transport)	*aceA, aceB, aceE, aceF, ackA, acnA, acnB, acs, adhE, agp, appB, appC, atpA, atpB, atpC, atpD, atpE, atpF, atpG, atpH, atpI, cydA, cydB, cydC, cydD, cyoA, cyoB, cyoC, cyoD, dld, eno, fba, fbp, fdhF, fdnG, fdnH, fdnI, fdoG, fdoH, fdoI, frdA, frdB, frdC, frdD, fumA, fumB, fumC, galM, gapA, gapC_1, gapC_2, glcB, glgA, glgC, glgP, glk, glpA, glpB, glpC, glpD, gltA, gnd, gpmA, gpmB, hyaA, hyaB, hyaC, hybA, hybC, hycB, hycE, hycF, hycG, icdA, lctD, ldhA, lpdA, malP, mdh, ndh, nuoA, nuoB, nuoE, nuoF, nuoG, nuoH, nuoI, nuoJ, nuoK, nuoL, nuoM, nuoN, pckA, pfkA, pfkB, pflA, pflB, pflC, pflD, pgi, pgk, pntA, pntB, ppc, ppsA, pta, purT, pykA, pykF, rpe, rpiA, rplB, sdhA, sdhB, sdhC, sdhD, sfcA, sucA, sucB, sucC, sucD, talB, tktA, tktB, tpiA, trxB, zwf, pgl* (30), *maeB* (30)
Alternative carbon source	*adhC, adhE, agaY, agaZ, aldA, aldB, aldH, araA, araB, araD, bglX, cpsG, deoB, fruK, fucA, fucI, fucK, fucO, galE, galK, galT, galU, gatD, gatY, glk, glpK, gntK, gntV, gpsA, lacZ, manA, melA, mtlD, nagA, nagB, nanA, pfkB, pgi, pgm, rbsK, rhaA, rhaB, rhaD, srlD, treC, xylA, xylB*
Amino acid metabolism	*adi, aldH, alr, ansA, ansB, argA, argB, argC, argD, argE, argF, argG, argH, argI, aroA, aroB, aroC, aroD, aroE, aroF, aroG, aroH, aroK, aroL, asd, asnA, asnB, aspA, aspC, avtA, cadA, carA, carB, cysC, cysD, cysE, cysH, cysI, cysJ, cysK, cysM, cysN, dadA, dadX, dapA, dapB, dapD, dapE, dapF, dsdA, gabD, gabT, gadA, gadB, gdhA, glk, glnA, gltB, gltD, glyA, goaG, hisA, hisB, hisC, hisD, hisF, hisG, hisH, hisI, ilvA, ilvB, ilvC, ilvD, ilvE, ilvG_1, ilvG_2, ilvH, ilvI, ilvM, ilvN, kbl, ldcC, leuA, leuB, leuC, leuD, lysA, lysC, metA, metB, metC, metE, metH, metK, metL, pheA, proA, proB, proC, prsA, putA, sdaA, sdaB, serA, serB, serC, speA, speB, speC, speD, speE, speF, tdcB, tdh, thrA, thrB, thrC, tnaA, trpA, trpB, trpC, trpD, trpE, tynA, tyrA, tyrB, ygjG, ygjH, alaB* (42), *dapC* (43), *pat* (44), *prr* (44), *sad* (45), *methylthioadenosine nucleosidase* (46), *5-methylthioribose kinase* (46), *5-methylthioribose-l-phosphate isomerase* (46), *adenosyl homocysteinase* (47), *L-cysteine desulfhydrase* (44), *glutaminase A* (44), *glutaminase* (44)
Purine & pyrimidine metabolism	*add, adk, amn, apt, cdd, cmk, codA, dcd, deoA, deoD, dgt, dut, gmk, gpt, gsk, guaA, guaB, guaC, hpt, mutT, ndk, nrdA, nrdB, nrdD, nrdE, nrdF, purA, purB, purC, purD, purE, purF, purH, purK, purL, purM, purN, purT, pyrB, pyrC, pyrD, pyrE, pyrF, pyrG, pyrH, pyrI, tdk, thyA, tmk, udk, udp, upp, ushA, xapA, yicP, CMP glycosylase* (48)
Vitamin & cofactor metabolism	*acpS, bioA, bioB, bioD, bioF, coaA, cyoE, cysG, entA, entB, entC, entD, entE, entF, epd, folA, folC, folD, folE, folK, folP, gcvH, gcvP, gcvT, gltX, glyA, gor, gshA, gshB, hemA, hemB, hemC, hemD, hemE, hemF, hemH, hemK, hemL, hemM, hemX, hemY, ilvC, lig, lpdA, menA, menB, menC, menD, menE, menF, menG, metF, mutT, nadA, nadB, nadC, nadE, ntpA, pabA, pabB, pabC, panB, panC, panD, pdxA, pdxB, pdxH, pdxJ, pdxK, pncB, purU, ribA, ribB, ribD, ribE, ribH, serC, thiC, thiE, thiF, thiG, thiH, thrC, ubiA, ubiB, ubiC, ubiG, ubiH, ubiX, yaaC, ygiG, nadD* (49), *nadF* (49), *nadG* (49), *panE* (50), *pncA* (49), *pncC* (49), *thiB* (51), *thiD* (51), *thiK* (51), *thiL* (51), *thiM* (51), *thiN* (51), *ubiE* (52), *ubiF* (52), *arabinose-5-phosphate isomerase* (22), *phosphopantothenate-cysteine ligase* (50), *phosphopantothenate-cysteine decarboxylase* (50), *phospho-pantetheine adenylyltransferase* (50), *dephosphoCoA kinase* (50), *NMN glycohydrolase* (49)
Lipid metabolism	*accA, accB, accD, atoB, cdh, cdsA, cls, dgkA, fabD, fabH, fadB, gpsA, ispA, ispB, pgpB, pgsA, psd, pssA, pgpA* (53)
Cell wall metabolism	*ddlA, ddlB, galF, galU, glmS, glmU, htrB, kdsA, kdsB, kdtA, lpxA, lpxB, lpxC, lpxD, mraY, msbB, murA, murB, murC, murD, murE, murF, murG, murI, rfaC, rfaD, rfaF, rfaG, rfaI, rfaJ, rfaL, ushA, glmM* (54), *lpcA* (55), *rfaE* (55), *tetraacyldisaccharide 4′ kinase* (55), *3-deoxy-o-manno-octulosonic-acid 8-phosphate phosphatase* (55)
Transport processes	*araE, araF, araG, araH, argT, aroP, artI, artJ, artM, artP, artQ, brnQ, cadB, chaA, chaB, chaC, cmtA, cmtB, codB, crr, cycA, cysA, cysP, cysT, cysU, cysW, cysZ, dctA, dcuA, dcuB, dppA, dppB, dppC, dppD, dppF, fadL, focA, fruA, fruB, fucP, gabP, galP, gatA, gatB, gatC, glnH, glnP, glnQ, glpF, glpT, gltI, gltK, gltL, gltP, gltS, gntT, gpt, hisJ, hisM, hisP, hisQ, hpt, kdpA, kdpB, kdpC, kgtP, lacY, lamB, livF, livG, livH, livJ, livK, livM, lldP, lysP, malE, malF, malG, malK, malX, manX, manY, manZ, melB, mglA, mglB, mglC, mtlA, mtr, nagE, nanT, nhaA, nhaB, nupC, nupG, oppA, oppB, oppC, oppD, oppF, panF, pheP, pitA, pitB, pnuC, potA, potB, potC, potD, potE, potF, potG, potH, potI, proP, proV, proW, proX, pstA, pstB, pstC, pstS, ptsA, ptsG, ptsI, ptsN, ptsP, purB, putP, rbsA, rbsB, rbsC, rbsD, rhaT, sapA, sapB, sapD, sbp, sdaC, srlA_1, srlA_2, srlB, tdcC, tnaB, treA, treB, trkA, trkG, trkH, tsx, tyrP, ugpA, ugpB, ugpC, ugpE, uraA, xapB, xylE, xylF, xylG, xylH, fruF* [56], *gnt5* (57), *metD* (43), *pnuE* (49), *scr* (56)

The *in silico E. coli* MG1655 metabolic genotype used herein is available on the web: http://gcrg.ucsd.edu/downloads.html.

(5–7). The metabolic capabilities of the *in silico* metabolic genotype were partially defined by mass balance constraints; mathematically represented by a matrix equation:

$$\mathbf{S}\cdot\mathbf{v} = 0. \qquad [1]$$

The matrix **S** is the mxn stoichiometric matrix, where m is the number of metabolites and n is the number of reactions in the network. The *E. coli* stoichiometric matrix was 436 × 720. The vector **v** represents all fluxes in the metabolic network, including the internal fluxes, transport fluxes, and the growth flux. The optimal **v** vector was determined and defined the steady-state metabolic flux distribution.

For the *E. coli* metabolic network, the number of fluxes was greater than the number of mass balance constraints; thus, there was a plurality of feasible flux distributions that satisfied the mass balance constraints (defined in Eq. 1), and the solutions (or feasible metabolic flux distributions) were confined to the nullspace of the matrix **S**.

In addition to the mass balance constraints, we imposed constraints on the magnitude of each individual metabolic flux.

$$\alpha_i \leq v_i \leq \beta_i. \qquad [2]$$

The linear inequality constraints were used to enforce the reversibility/irreversibility of metabolic reactions and the max-

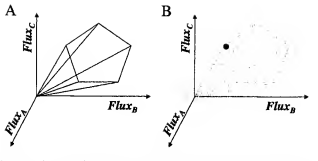

Fig. 1. The feasible solution set for a hypothetical metabolic reaction network. (*A*) The steady-state operation of the metabolic network is restricted to the region within a cone, defined as the feasible set (*B*). The feasible set contains all flux vectors that satisfy the physicochemical constrains (Eqs. 1 and 2). Thus, the feasible set defines the capabilities of the metabolic network. All feasible metabolic flux distributions lie within the feasible set, and (*B*) in the limiting case, where all constraints on the metabolic network are known, such as the enzyme kinetics and gene regulation, the feasible set may be reduced to a single point. This single point must lie within the feasible set.

imal metabolic fluxes in the transport reactions. The intersection of the nullspace and the region defined by the linear inequalities formally defined a region in flux space that we will refer to as the feasible set. The feasible set defined the capabilities of the metabolic network subject to the subset of cellular constraints, and all feasible metabolic flux distributions lie within the feasible set (see Fig. 1). However, every vector **v** within the feasible set is not reachable by the cell under a given condition because of other constraints not considered in the analysis (i.e., maximal internal fluxes and gene regulation). The feasible set can be further reduced by imposing additional constraints, and if all of the necessary details to describe metabolic dynamics are known, then the feasible set may reduce to a small region or even a single point (see Fig. 1).

For the analysis presented herein, we defined $\alpha_i = 0$ for irreversible internal fluxes, and $\alpha_i = -\infty$ for reversible internal fluxes. The reversibility of the metabolic reactions was determined from the biochemical literature and is identified for each reaction on the web site. The transport flux for inorganic phosphate, ammonia, carbon dioxide, sulfate, potassium, and sodium was unrestrained ($\alpha_i = -\infty$ and $\beta_i = \infty$). The transport flux for the other metabolites, when available in the *in silico* medium, was constrained between zero and the maximal level ($0 < v_i < v_i^{max}$). However, when the metabolite was not available in the medium, the transport flux was constrained to zero. The transport flux for metabolites that were capable of leaving the metabolic network (i.e., acetate, ethanol, lactate, succinate, formate, pyruvate, etc.) always was unconstrained in the outward direction.

A particular metabolic flux distribution within the feasible set was found by using linear programming (LP). A commercially available LP package was used (LINDO, Lindo Systems, Chicago). LP identified a solution that minimized a particular metabolic objective (subject to the imposed constraints) (5, 25, 26), and was formulated as shown. Minimize $-Z$, where

$$Z = \Sigma c_i \cdot v_i = \langle \mathbf{c} \cdot \mathbf{v} \rangle. \qquad [3]$$

The vector **c** was used to select a linear combination of metabolic fluxes to include in the objective function (27). Herein, **c** was defined as the unit vector in the direction of the growth flux, and the growth flux was defined in terms of the biosynthetic requirements:

$$\sum_{all\ m} d_m \cdot X_m \xrightarrow{v_{growth}} \text{Biomass}, \qquad [4]$$

where d_m is the biomass composition of metabolite X_m (defined from the literature; ref. 28), and the growth flux is modeled as a single reaction that converts all of the biosynthetic precursors into biomass.

Results

FBA was used to examine the change in the metabolic capabilities caused by gene deletions. To simulate a gene deletion, the flux through the corresponding enzymatic reaction was restricted to zero. Genes that code for isozymes or genes that code for components of same enzyme complex were simultaneously removed (i.e., *aceEF, sucCD*). The optimal value of the objective (Z_{mutant}) was compared with the "wild-type" objective (Z) to determine the systemic effect of the gene deletion. The ratio of optimal growth yields (Z_{mutant}/Z) was calculated (Fig. 2).

Gene Deletions. *E. coli* MG1655 *in silico* was subjected to deletion of each individual gene product in the central metabolic pathways [glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, respiration processes], and the maximal ca-

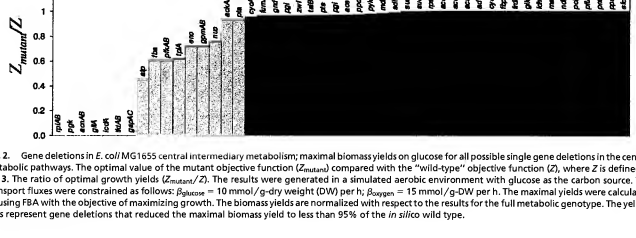

Fig. 2. Gene deletions in *E. coli* MG1655 central intermediary metabolism; maximal biomass yields on glucose for all possible single gene deletions in the central metabolic pathways. The optimal value of the mutant objective function (Z_{mutant}) compared with the "wild-type" objective function (Z), where Z is defined in Eq. 3. The ratio of optimal growth yields (Z_{mutant}/Z). The results were generated in a simulated aerobic environment with glucose as the carbon source. The transport fluxes were constrained as follows: $\beta_{glucose} = 10$ mmol/g-dry weight (DW) per h; $\beta_{oxygen} = 15$ mmol/g-DW per h. The maximal yields were calculated by using FBA with the objective of maximizing growth. The biomass yields are normalized with respect to the results for the full metabolic genotype. The yellow bars represent gene deletions that reduced the maximal biomass yield to less than 95% of the *in silico* wild type.

pability of each *in silico* mutant metabolic network to support growth was assessed with FBA. The simulations were performed under an aerobic growth environment on minimal glucose medium.

The results identified the essential (required for growth) central metabolic genes (Fig. 2). For growth on glucose, the essential gene products were involved in the three-carbon stage of glycolysis, three reactions of the TCA cycle, and several points within the PPP. The remainder of the central metabolic genes could be removed and *E. coli in silico* maintained the potential to support cellular growth. This result was related to the interconnectivity of the metabolic reactions. The *in silico* gene deletion results suggest that a large number of the central metabolic genes can be removed without eliminating the capability of the metabolic network to support growth under the conditions considered.

Are the *in Silico* Redundancy Results Consistent with Mutant Data? The *in silico* gene deletion study results were compared with growth data from known mutants. The growth characteristics of a series of *E. coli* mutants on several different carbon sources were examined and compared with the *in silico* deletion results (Table 2). From this analysis, 86% (68 of 79 cases) of the *in silico* predictions were consistent with the experimental observations.

How Are Cellular Fluxes Redistributed? The potential of many *in silico* deletion strains to support growth led to questions regarding how the *E. coli* metabolic genotype deals with the loss of metabolic functions. The answer involves the degree of stoichiometric connectivity of key metabolites. For illustration, the flux redistributions to optimally support growth of a single mutant and a double mutant were investigated.

The optimal metabolic flux distribution for the *in silico* wild type was calculated (Fig. 3). The constraints used in the LP problem are defined in the figure legend. The *in silico* results suggest that optimally the oxidative branch of the PPP was used to generate a large fraction of the NADPH (66% *in silico*: 20–50% reported in the literature, ref. 29), and the TCA cycle produced NADH. The optimal flux distribution also suggested that the majority of the high-energy phosphate bonds were generated via oxidative phosphorylation and acetate secretion because of limitations of the oxygen supply.

The *in silico* gene deletion results predicted that the optimal biomass yield of the *zwf* (glucose-6-phosphate dehydrogenase) *in silico* strain was slightly less than the wild type. The optimal flux distribution of the *zwf in silico* strain (Fig. 2) was calculated, and the NADPH was optimally generated through the transhydrogenase reaction and an elevated TCA cycle flux. The PPP biosynthetic precursors were generated in the nonoxidative branch. This metabolic flux rerouting resulted in an optimal biomass yield that was 99% of the *in silico* wild type.

The transhydrogenase (*pnt*) also was deleted *in silico*, creating an *in silico* double deletion mutant and eliminating an alternate source of NADPH. The double mutant still maintained growth potential. The optimal flux distribution (Fig. 2) used the isocitrate dehydrogenase and the malic enzyme to produce NADPH. The optimal biomass yield of the double mutant was 92% of the *in silico* wild type. The FBA results were consistent with the experimental observations that the *zwf* strain (30) and the *pnt* strain (29) are able to grow at near wild-type yields. Furthermore, the *zwf pnt* double mutant strain also has been shown to grow ($\mu_{\text{mutant}}/\mu_{\text{wild type}} = 57\%$) (29).

Discussion

Extensive information about the molecular composition and function of several single-cellular organisms has become available. A next important step will be to incorporate the available information to generate whole-cell models with interpretative and predictive capability. Herein, we have taken a step in that direction by using a set of constraints on cellular metabolism on the whole-cell level to analyze the metabolic capabilities of the

Table 2. Comparison of the predicted mutant growth characteristics from the gene deletion study to published experimental results with single mutants

Gene	glc	gl	succ	ac	Reference
aceA	+/+		+/+	−/−	(58)
aceB				−/−	(58)
*aceEF**	−/+				(60)
ackA				+/+	(61)
acn	−/−			−/−	(58)
acs				+/+	(61)
cyd	+/+				(62)
cyo	+/+				(62)
eno†	−/+	−/+	−/−	−/−	(30)
fba‖	−/+				(30)
fbp	+/+	−/−	−/−	−/−	(30)
frd	+/+		+/+	+/+	(60)
gap	−/−	−/−	−/−	−/−	(30)
glk	+/+				(30)
gltA	−/−			−/−	(58)
gnd	+/+				(30)
idh	−/−			−/−	(58)
mdh††	+/+	+/+	+/+		(63)
ndh	+/+	+/+			(59)
nuo	+/+	+/+			(59)
pfk†	−/+				(30)
pgi‡	+/+	+/−	+/−		(30)
pgk	−/−	−/−	−/−	−/−	(30)
pgl	+/+				(30)
pntAB	+/+	+/+	+/+		(29)
ppc§	±/+	−/+	+/+		(63, 64)
pta				+/+	(61)
pts	+/+				(30)
pyk	+/+				(30)
rpi	−/−	−/−	−/−	−/−	(30)
sdhABCD	+/+		−/−	−/−	(58)
sucAB	+/+		−/+	−/+	(60)
tktAB	−/−				(30)
*tpi***	−/+	−/−	−/−	−/−	(30)
unc	+/+		±/+	−/−	(66–68)
zwf	+/+	+/+	+/+		(30)

Results are scored as + or − meaning growth or no growth determined from *in vivo/in silico* data. The ± indicates that suppressor mutations have been observed that allow the mutant strain to grow. In 68 of 79 cases the *in silico* behavior is the same as the experimentally observed behavior. glc, glucose; ac, acetate; gl, glycerol; succ, succinate.

*The *in vivo aceAE* strain is able to grow under anaerobic growth conditions by using the pyruvate formate lyase.

†The *in silico pfk* strain is able to grow by increasing the PPP flux ≈ 5× and using the *pps* gene product to overcome PEP deficiency.

‡The *in silico pgi* strain is unable to grow with glycerol or succinate as the carbon source because it is unable to synthesize glycogen and one carbohydrate component in the lipopolysaccharide. These are likely nonessential components of the biomass.

§The grow on glycerol and glucose is possible through the utilization of the glyoxylate bypass. Constitutive mutations in the glyoxylate bypass can suppress the *ppc* phenotype.

¶The *in silico eno* strain is able to grow by the synthesis and degradation of serine.

‖There is evidence that *fba* has an inhibitory effect on stable RNA synthesis (65). Such an inhibition cannot be predicted by FBA.

**The inability of *tpi* mutants to grow on glucose may be related to the accumulation of dihydroxyacetone phosphate, which leads to the formation of the bactericidal compound methylglyoxal (30).

††Very slow growth on glycerol and succinate.

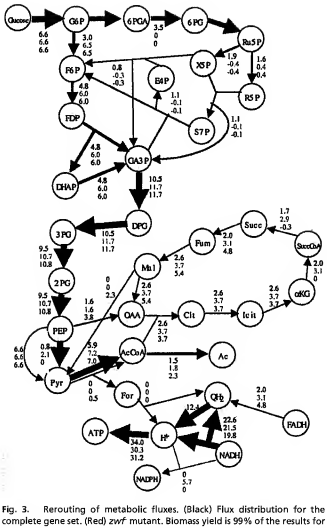

Fig. 3. Rerouting of metabolic fluxes. (Black) Flux distribution for the complete gene set. (Red) *zwf* mutant. Biomass yield is 99% of the results for the full metabolic genotype. (Blue) *zwf pnt* mutant. Biomass yield is 92% of the results for the full metabolic genotype (see text). The solid lines represent enzymes that are being used, with the corresponding flux value noted. The fluxes [substrates converted/h per g-dry weight (DW)] were calculated by using FBA with the input parameters of glucose uptake rate ($\beta_{glucose}$ = 6.6 mmol glucose/h per g-DW) and oxygen uptake rate (β_{oxygen} = 12.4 mmol oxygen/h per g-DW) (41).

extensively studied bacterium *E. coli*. We have calculated the optimal metabolic network utilization with a FBA. The *in silico* results, based only on stoichiometric and capacity constraints, were consistent with experimental data for the wild type and many of the mutant strains examined.

The construction of comprehensive *in silico* metabolic maps provided a framework to study the consequences of alterations in the genotype and to gain insight into the genotype-phenotype relation. The stoichiometric matrix and FBA were used to analyze the consequences of the loss of a gene product function on the metabolic capabilities of *E. coli*. The results demonstrated an important property of the *E. coli* metabolic network, namely that there are relatively few critical gene products in central metabolism. The nonessential genes in several organisms have been found experimentally on a genome scale (31, 32), which opens up the opportunity to critically test the *in silico* predictions. The *in silico* analysis also suggests that although the ability to grow in one defined environment is only slightly altered the ability to adjust to different environments may be diminished (33). Therefore, the *in silico* analysis provides a methodology for relating the specific biochemical function of the metabolic enzymes to the integrated properties of the metabolic network.

The *in silico* analysis presented herein is not the typical metabolic modeling; more appropriately, the analysis can be thought of as a constraining approach. This approach defines the "best" the cell can do and identifies what the cell cannot do, rather than attempting to predict how the cell actually will behave under a given set of conditions. To accomplish this, we have used a set of physicochemical constraints for which there is reliable information available, in particular the stoichiometric properties. FBA does not directly consider regulation or the regulatory constraints on the metabolic network.

The results of FBA can be interpreted in a qualitative or a quantitative sense. At the first level we can ask whether a cell is able to grow under given circumstances and how a loss of the function of a gene product influences this ability. The results presented herein fall into this category. Quantitative predictions would hold true if the cell optimized its growth under the growth conditions considered. Therefore, when applying LP to predict quantitatively the optimal metabolic pathway utilization, it is assumed that the cell has found an "optimal solution" for survival through natural selection, and we have equated survival with growth. Although *E. coli* may grow optimally in defined media, one should not expect that optimizing growth is the governing objective of the cell under all growth conditions. For example, the regulatory mechanisms can only evolve to stoichiometric optimality in a condition to which the cell has been exposed. Furthermore, the growth behavior of mutant strains is unlikely to be optimal. However, FBA can still be used to delineate the metabolic capabilities of mutant cells based on constraining features, because both wild-type and mutant cells must obey the physicochemical constraints imposed.

The constraints on the system accurately reflect the steady-state capabilities of the metabolic network, but does the calculated optimal flux vector in the feasible set accurately reflect the behavior of the actual metabolic network? It has been shown that in a minimal media the metabolic behavior of wild-type *E. coli* is consistent with stoichiometric optimality (34). Furthermore, more detailed and critical experimental results are consistent with the hypothesis that *E. coli* does optimize its growth in acetate or succinate minimal media (33). Taken together these results call for critical experimental investigation to evaluate the hypothesis that stoichiometric and capacity constraints are the principal constraints that limit *E. coli* maximal growth. Even though growth and metabolic behavior in minimal media are consistent with FBA results, one still must determine the generality of optimal performance. The call for critical experimentation is particularly timely, given the increasing number of genome scale measurements that are now possible through two-dimensional gels (35, 36) and DNA array technology (37, 38). Furthermore, the ability to precisely remove ORFs can be used to design critical experiments (39). The *in silico* model can be used to choose the most informative knockouts and to design growth experiments with the knockouts.

At the present time, the annotation of the *E. coli* genome is incomplete, and about one-third of its ORFs do not have a functional assignment. Thus, the metabolic genotype studied here may lack some metabolic capabilities that *E. coli* possesses. The biochemical literature also was used to define the *in silico* metabolic genotype, and given the long history of *E. coli* metabolic research (20), a large percentage of the *E. coli* metabolic capabilities likely have been identified. However, if additional metabolic capabilities are discovered (40), the *E. coli* stoichiometric matrix can be updated, leading to an iterative model building process. Additionally, the *in silico* analysis can help identify missing or incorrect functional assignments by

identifying sets of metabolic reactions that are not connected to the metabolic network by the mass balance constraints.

The ability to analyze, interpret, and ultimately predict cellular behavior has been a long sought-after goal. The genome sequencing projects are defining the molecular components within the cell, and describing the integrated function of these molecular components will be a challenging task. The results presented herein suggest that it may be possible to analyze cellular metabolism based on a subset of the constraining features. Continued prediction and experimental verification will be an integral part in the further development of *in silico* strains. Deciphering the complex relation between the genotype and the phenotype will involve the biological sciences, computer science, and quantitative analysis, all of which must be included in the bioengineering of the 21st century.

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DMEM. H1299 cells were a gift from J. Chen and were grown in RPMI. All transfections were carried out using Lipofectamine 2000 (Invitrogen), Oligofectamine (Invitrogen) or Genepporter 2 (Gene Therapy Systems) according to manufacturer's recommendations. To assess the effect of COP1 on steady-state levels of p53, Saos-2 cells were transfected with increasing amounts of Flag–COP1 or Flag–COP1ΔRING with 250 ng pcDNA3.1+p53, and U2-OS cells were transfected with or without increasing amounts (0.5, 1 and 2 μg) of pCMV–Flag–COP1 or pCMV–Flag–COP1ΔRING and treated with 50 μM ALLN for 6 h before cell collection where indicated. For reporter assays, Saos-2 or H1299 cells were transiently transfected with 150 ng of pcDNA3.1+p53 or pcDNA3.1+p53R175H, 100 ng of *p21–luc*, *bax–luc*, *COP1–luc*, *COP1mut–luc* or NS–luc, and 10 ng of pCMV–β–Gal, with or without increasing amounts (0.5, 1 and 2 μg) of pCMV–Flag–COP1 or pCMV–Flag–COP1ΔRING. Luciferase assays were carried out according to manufacturer's instructions (Promega). For p53-induced cell-death assays, Saos-2 cells were transiently transfected with 1 μg of enhanced green fluorescent protein (EGFP) and 5 μg of pcDNA3.1+, pcDNA3.1+p53, pCMV–Flag–COP1 or pcDNA3.1+p53, and 15 μg of pCMV–Flag–COP1 for 48 h. Cells were harvested and stained with propidium iodide for analysis by fluorescence-activated cell sorting (FACS). COP1 siRNA1 (AACUACCAA GAUAACCUUGA), COP1 siRNA1 inverted (AAAGUUCCAAUAGAACAACGUC), COP1 siRNA2 (AAGACUUGGAGCAGUGUUACU), COP1 siRNA3 (AAGAGGUGUUGGAG UGUUGAC), Pirh2 siRNA1 (AACTGTGGAATTTGTAGG), Pirh2 B inverted (AAGGAUGUUAAGGUGUCAA), Pirh2 siRNA2 (AAUGUAACUUAUGCCUAGCUA), Pirh2 siRNA2 inverted (AAAUCGAUCCGUAUUCAUGU) and MDM2 (AAGGAAUUUAGACAACCUGAA) siRNA oligonucleotides with 3′ dTdT overhangs were synthesized by Genentech or Dharmacon. Control siRNA in experiments refers to a mixture of inverted siRNA oligonucleotides. U2-OS, H1299, Saos-2 and BJ cells were transfected with siRNA oligonucleotides three times at 24–36 h intervals and expanded as necessary to prevent contact inhibition.

Immunoprecipitation, GST pull-down assays and pulse–chase analysis

Cells were lysed in immunoprecipitation (IP) lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4, and protease inhibitor mix) or radioimmunoprecipitation assay (RIPA buffer (0.1% SDS, 1% NP-40, 150 mM NaCl, 0.5% deoxycholate, 50 mM Tris, pH 7.4, and protease inhibitor mix), pre-cleared and immunoprecipitated with target antibody and protein A/G PLUS beads. Identification of COP1-interacting proteins was carried out as previously described[13], except that U2-OS cells stably expressing Flag–COP1 were generated. GST pull-down assays were carried out with GST or GST–p53 combined with *in vitro* translated HA–COP1 in PBST (PBS with 0.1% Tween 20) and incubated on ice for 1 h. GST-bound proteins were subject to SDS–PAGE and immunoblot with anti-HA and anti-GST. IPs were washed in lysis buffer with high salt as required. Pulse–chase experiments were carried out as previously described[13], except that HEK293T cells were transfected with pCMV–Flag6x or pCMV–Flag–COP1 for 24 h, and U2-OS cells were transfected with siRNA oligonucleotides as indicated.

In vitro ubiquitination assays

For *in vitro* ubiquitination reactions, *in vitro*-translated p53 was immunoprecipitated with anti–p53 (DO-1 and FL-393) and washed five times with IP lysis buffer, and reactions were carried out on protein A/G beads. Ten micrograms of Flag–ubiquitin (Sigma), 20 ng of UbcH5b (A.G. Scientific), 20 ng of rabbit E1 (Sigma) and 500 ng of GST–COP1 (E3), which was pre-incubated with 20 μM $ZnCl_2$ for 30 min at room temperature, were incubated in a buffer containing 50 mM Tris, pH 7.5, 2 mM ATP, 5 mM $MgCl_2$, 20 μM $ZnCl_2$ and 2 mM DTT. After incubation for 2 h at 30 °C with gentle agitation, reactions were boiled in PBST with 1% SDS for 5 min and reduced to 0.1% SDS with PBST for re-immunoprecipitation with anti-p53 (DO-1 and FL-393). Finally, samples were subjected to SDS–PAGE followed by immunoblotting with anti-Flag–HRP (M2) to detect ubiquitinated species of p53.

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Integrating high-throughput and computational data elucidates bacterial networks

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The flood of high-throughput biological data has led to the expectation that computational (or *in silico*) models can be used to direct biological discovery, enabling biologists to reconcile heterogeneous data types, find inconsistencies and systematically generate hypotheses[1–3]. Such a process is fundamentally iterative, where each iteration involves making model predictions, obtaining experimental data, reconciling the predicted outcomes with experimental ones, and using discrepancies to update the *in silico* model. Here we have reconstructed, on the basis of information derived from literature and databases, the first integrated genome-scale computational model of a transcriptional regulatory and metabolic network. The model accounts for 1,010 genes in *Escherichia coli*, including 104 regulatory genes whose products together with other stimuli regulate the expression of 479 of the 906 genes in the recon-

structed metabolic network. This model is able not only to predict the outcomes of high-throughput growth phenotyping and gene expression experiments, but also to indicate knowledge gaps and identify previously unknown components and interactions in the regulatory and metabolic networks. We find that a systems biology approach that combines genome-scale experimentation and computation can systematically generate hypotheses on the basis of disparate data sources.

We first validated the model, or '*in silico* strain' of *E. coli* (*i*MC1010^{v1}; see ref. 4 for conventions for naming *in silico* strains), against a data set of 13,750 growth phenotypes[5] obtained from the ASAP database[6], and then used this genome-scale model to select

a

Exp/Met/Reg	Percentage
Reg and Met models predict correctly	
+/+/+ 6,222	45.3%
–/–/– 2,094	15.2%
Reg model only predicts correctly	
–/+/+ 657	4.8%
+/–/+ 0	0%
+/n/+ 1,350	9.8%
–/n/– 505	3.7%
Met model only predicts correctly	
+/n/– 242	1.8%
–/n/+ 153	1.1%
–/+/+ 0	0%
+/+/– 257	1.9%
Neither model predicts correctly	
–/+/+ 702	5.1%
+/–/– 1,568	11.4%
Met 8,968	65.2%
Reg 10,828	78.7%
Total 13,750	100%

c

Carbon sources	FA	Nitrogen sources	FA
● Acetoacetic acid	0.42	● Adenine	0.42
● Formic acid	0.05	○ Alanine–Leucine	0.28
○ γ-Amino butyric acid	0.21	Guanosine	0.51
● Glycine	0.51	ⓚ L-Lysine	0.44
○ L-Arginine	0.35	ⓜ L-Methionine	0.42
L-Glutamic acid	0.30	Ⓛ L-Phenylalanine	0.24
○ L-Ornithine	0.21	● *N*-Acetyl-D-mannosamine	0.48
○ Putrescine	0.41	● Putrescine	0.60
ⓣ Thymidine	0.00	ⓧ Xanthine	0.04

Knockout strains	FA	Knockout strains	FA
Δ*argB*	0.39	ⓘ Δ*ilvY*	0.45
Δ*argC*	0.31	ⓜ Δ*metA*	0.39
Δ*argD*	0.27	Δ*pgi*	0.30
Δ*argE*	0.52	ⓟ Δ*pls*	0.28
Δ*argG*	0.57	Ⓒ Δ*purD*	0.30
ⓐ Δ*glgA*	0.25	ⓡ Δ*purH*	0.41
ⓖ Δ*glgC*	0.25	Δ*tpiA*	0.50
Ⓓ Δ*ilvD*	0.52		

○ Missing regulatory interactions
Ⓜ Missing metabolic enzymes
● Conflicts with subsequent observations

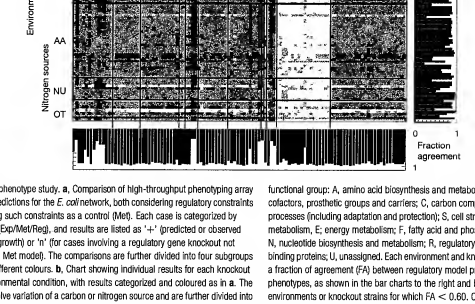

Figure 1 Growth phenotype study. **a**, Comparison of high-throughput phenotyping array data (Exp) with predictions for the *E. coli* network, both considering regulatory constraints (Reg) and ignoring such constraints as a control (Met). Each case is categorized by comparison type (Exp/Met/Reg), and results are listed as '+' (predicted or observed growth), '–' (no growth) or 'n' (for cases involving a regulatory gene knockout not predictable by the Met model). The comparisons are further divided into four subgroups represented by different colours. **b**, Chart showing individual results for each knockout under each environmental condition, with results categorized and coloured as in **a**. The environments involve variation of a carbon or nitrogen source and are further divided into subgroups: AA, amino acid or derivative; CM, central metabolic intermediate; NU, nucleotide or nucleoside; SU, sugar; OT, other. The knockout strains are also divided by functional group: A, amino acid biosynthesis and metabolism; B, biosynthesis of cofactors, prosthetic groups and carriers; C, carbon compound catabolism; P, cell processes (including adaptation and protection); S, cell structure; M, central intermediary metabolism; E, energy metabolism; F, fatty acid and phospholipid metabolism; N, nucleotide biosynthesis and metabolism; R, regulatory function; T, transport and binding proteins; U, unassigned. Each environment and knockout strain is associated with a fraction of agreement (FA) between regulatory model predictions and observed phenotypes, as shown in the bar charts to the right and below. **c**, Table showing all environments or knockout strains for which FA < 0.60. Of these substrates or knockout strains, 18 point to uncharacterized metabolic or regulatory capabilities in this organism, as indicated (see Supplementary Information for a case-by-case analysis).

transcription factors for prospective gene knockout studies. Comparison with the growth phenotypes showed that experimental and computational outcomes agreed in 10,828 (78.7%) of the cases examined, which is roughly the same success rate achieved in previous studies in *E. coli* and yeast that considered only a few hundred phenotypes[7–9]. In addition, 2,512 (18.3%) of the cases were predicted correctly only when regulatory effects were incorporated into the metabolic model (see Supplementary Information for details).

The comparisons in this study identified several substrates and knockout strains whose growth behaviour did not match predictions (Fig. 1). Further investigation of these conditions and strains led to the identification of five environmental conditions in which dominant, as yet uncharacterized, regulatory interactions actively contribute to the observed growth phenotype, and five environmental conditions and eight knockout strains that highlight uncharacterized enzymes or non-canonical pathways that are predicted to be used by the organism (Fig. 1; a detailed analysis of the discrepancies is provided in the Supplementary Information).

We wanted to determine the utility of this model-driven approach in elucidating transcriptional regulatory networks. A previous study, which evaluated the consistency between existing gene expression data sets and the known transcriptional regulatory network of *E. coli*, identified the response to oxygen deprivation as a partially consistent module[10,11]. We therefore targeted this part of the transcriptional regulatory network for further network characterization. Six strains with knockouts of key transcriptional regulators in the oxygen response (Δ*arcA*, Δ*appY*, Δ*fnr*, Δ*oxyR*, Δ*soxS* and the double knockout Δ*arcA*Δ*fnr*) were constructed. The messenger RNA expression profiles of these strains, as well as the

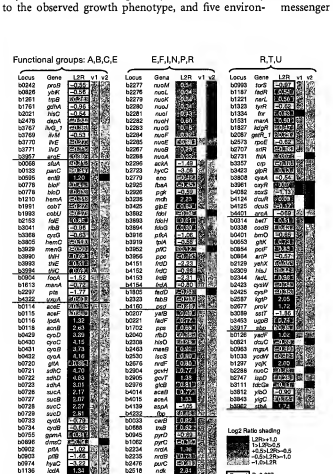

Figure 2 Characterization of the regulatory network related to the aerobic–anaerobic shift. **a**, The locus numbers, gene names and the log2 ratio (L2R) of gene expression (aerobic to anaerobic) are shown for all model genes with either predicted or observed changes in expression (genes were divided into the same functional groups as in Fig. 1). The L2R values are shaded depending on the magnitude of the expression shift, and those enclosed by a box indicate a statistically significant change in expression ($P < 0.007$, FDR < 5%). Comparisons between the experimental data and model predictions are also shown, where v1 (iMC1010^{v1}) and v2 (iMC1010^{v2}) designate the model used in the predictions. Filled and open symbols indicate model predictions and experimental data, respectively; rectangles indicate no change in gene expression; triangles indicate a change in expression, as well as the direction of change (upregulated or downregulated). **b**, Comparison of the predicted and observed expression changes for the v1 and v2 models. A question mark indicates either that the given gene was not included in the model or that no expression data were obtained for a given shift; other symbols are the same as in **a**. **c**, Systematic perturbation analysis was used to determine the transcription factors responsible for the expression change. The transcription factors knocked out in the six strains are shown on top. Each row indicates a pattern of knockout strains in which differential expression was abolished. The number of genes that show this pattern is indicated on the right. Thus, the first row indicates that for 73 of the 437 genes that showed differential expression in the wild-type strain (or 20 of the 151 genes accounted for by the model), the observed differential expression was abolished only in the Δ*arcA*Δ*fnr* knockout strain.

wild-type strain, were measured in aerobic and anaerobic glucose minimal medium conditions. The data were analysed[12] in the context of *i*MC1010^{v1} predictions to identify new interactions in the regulatory network (Fig. 2).

Expression profiling of the wild-type strain identified 437 genes that experienced a significant change in transcription in response to oxygen deprivation (*t*-test, multiple testing corrected to give a false discovery rate (FDR) of less than 5%); of these, 151 genes were included in *i*MC1010^{v1}. Computationally, 75 genes were predicted by *i*MC1010^{v1} to show differential expression in response to oxygen deprivation. These 75 genes could be divided into three categories: 23 agreed with measured expression changes; 24 had a predicted expression change that was either not found to be statistically significant in the experimental data (23/24) or in a direction opposite to that of the experimental data (1/24); and for 28 genes there were no expression data available (transcript abundance was determined to be 'absent' for two or more of the replicates). Thus, of the 47 (=23 + 24) differentially expressed genes that could be compared between the model computation and experiment, 23 (or 49% accuracy) agreed. Considering the overall number of genes in the model for which there were experimental data, the overlap (23) between the sets of predicted (47) and experimentally detected (151) differentially expressed genes is significant in comparison to a model that would randomly predict expression changes ($P < 0.005$ on the basis of a cumulative binomial distribution). There were 151 genes that were differentially expressed and included in the model; however, with only 23 (or 15% coverage) correctly computed, there is much room for expanding the transcriptional regulatory network in *i*MC1010^{v1} on the basis of the experimental data (Fig. 3).

To understand which transcription factors are involved in regulating these differentially expressed genes after oxygen deprivation, we compared the gene expression data for the wild-type and each knockout strain separately. Using two-way analysis of variance (ANOVA), we could determine whether the differential expression was significantly altered in the knockout strain as compared with the wild type. A large portion of the expression changes observed for the wild-type strain were not significantly affected in any of the knockout strains (195/437 or 44.6% of genes overall, 63/151 or 41.7% of genes in the model, FDR < 5%), suggesting that none of the five transcription factors studied here regulates the expression of these genes or that combinatorial interactions between multiple transcription factors are involved in regulation. For the remainder of the genes, differential expression was abolished in one or more of the knockout strains (Fig. 2c).

The ANOVA-based identification of transcription factors that influence differential expression of specific genes enabled us systematically to rewrite, relax or remove various regulatory rules in the model to resolve the discrepancies between *i*MC1010^{v1} and the experimentally determined wild-type differential gene expression. For many (81) of the genes, a regulatory rule already existed and had to be reconciled with our new data to accommodate the newly determined transcription factor dependencies. For genes where none of the knockouts abolished differential expression, we simply based a new regulatory rule on the presence of oxygen rather than a transcription factor (39 genes). By contrast, for genes where a change in expression was predicted but not observed, we removed oxygen dependency from the existing regulatory rule (23 genes). In addition, for 12 genes the predicted expression changes agreed with the observed expression in the wild type, but our knockout perturbation analysis indicated that the transcription factors involved in the regulation differed from previously reported data and the model needed to be changed (all new regulatory rules are detailed in the Supplementary Information).

The updated model (*i*MC1010^{v2}) was used to recalculate all of the predictions for both the aerobic and anaerobic expression data and the high-throughput phenotyping arrays. Note that *i*MC1010^{v2} accounts for the same genes as *i*MC1010^{v1} but has different regulatory interactions among the gene products and oxygen as an environmental variable. We found agreement between model predictions and the gene expression data to be substantially higher using the *i*MC1010^{v2} model, as expected (Fig. 2c). Specifically, 100 of the 151 expression changes were correctly computed with *i*MC1010^{v2}, and the number of false-positive predictions (Fig. 2, yellow boxes) was reduced to zero. In resolving many of the cases of unpredicted differential expression (Fig. 2, orange boxes), we found that implementation of the ANOVA-derived rule resulted in the inability of the wild-type or knockout *in silico* strain to grow aerobically or anaerobically on glucose, or under other conditions where growth had been previously established (for example, wild-type and knockout strain average growth rate under aerobic conditions, 0.68 ± 0.04 per hour; anaerobic, 0.43 ± 0.07 per hour). Such cases may be thought of as an 'overfit' of the microarray data. Accordingly, we relaxed the regulatory rule for these genes (42 in total) to allow for a correct phenotype prediction. Comparisons for the high-throughput phenotyping data revealed very little difference from Fig. 1 (only 11 out of 13,750 cases were affected; see Supplementary Information).

The iterative modification of the regulatory rules led to three main observations. First, some of the results of the knockout perturbation analysis are complex enough to make boolean rule formulation difficult. For example, the interplay of Fnr and ArcA can lead to complex behaviours where the expression change observed in wild type is abolished in the Δ*arcA* or the Δ*fnr* strains, but not in the Δ*arcA*Δ*fnr* strain. Such complex interplay among transcription factors can lead to specialized expression changes, as observed in the *cydAB* response to anaerobic, microaerobic and aerobic conditions[13,14].

Second, in revising regulatory rules for transcription factors we found that whereas in some cases, such as *arcA*, expression of a regulatory protein correlates positively with its activity, in several

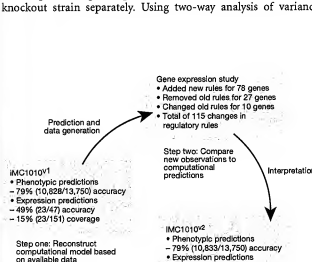

Figure 3 Biological network elucidation by a model-centric approach. Metabolic and regulatory networks may be expanded by using high-throughput phenotyping and gene expression data coupled with the predictions of a computational model. If model predictions are consistent with experimental observations, the network is adequately characterized. If not, the model identifies a knowledge gap and may be used to update, validate and generate hypotheses about organism function. Accuracy refers to the percentage of model predictions that agree with experimental data; coverage indicates the percentage of experimental changes predicted correctly by the model.

cases, including *fnr*, *betI* and *fur* among others, the mRNA level of a regulatory gene is reduced when the protein is in fact activated. For example, under anaerobic conditions when Fnr is known to be active[13], its expression is significantly reduced. Such behaviour, underscored by similar observations of mRNA transcript levels and corresponding protein product abundance in yeast[15], suggests that the identification of regulatory networks, and transcription factors involved in regulation in particular, will not be accomplished by the determination of co-regulated gene sets alone.

Third, many of these gene expression changes involve complex interactions and indirect effects. Transcription factors may be affected, for example, by the presence of fermentation by-products or the build up of internal metabolites. Such effects would be extremely difficult to identify or account for without a computational model.

In summary, we find that the reconciliation of high-throughput data sets with genome-scale computational model predictions enables systematic and effective identification of new components and interactions in microbial biological networks. Our study illustrates only the first round of an iterative model building strategy where an initial model based on literature-derived information (iMC1010[v1]) is used to design informative experiments and then updated on the basis of the new experimental data obtained (iMC1010[v2]). Another round of perturbation experiments will lead to iMC1010[v3], and so on. We expect that after an effort of some years and many iterations of this process, regulatory network elucidation for *E. coli* will be essentially complete. □

Methods

Computational model

We constructed the model of the *E. coli* metabolic and regulatory network by identifying network components, their functions and interactions from the primary literature[4,9,16]. Many approaches have been developed to analyse large-scale metabolic[17–20] and transcriptional regulatory[21–26] networks. Growth and gene expression simulations were done by regulated flux-balance analysis, which combines linear optimization to determine a growth-optimized metabolic flux distribution with logic statements to simulate the effects of regulatory processes over time. The whole model construction and simulation process has been described elsewhere in detail[18].

Strains and culture

The parent strain for knockout strains in this study was K-12 MG1655 (ref. 27), and all deletion strains were generated as described[28]. Growth experiments for the gene expression study were done on M9 glucose medium (2 g l^{-1}) under aerobic and anaerobic conditions, as described[22]. The growth data contained in the ASAP database were obtained by using high-throughput phenotype arrays (Biolog)[8]. In some cases (where the viability of a particular environment was unclear from the phenotype array data), we cross-validated the ASAP phenotyping data by culturing the wild-type strain under the given conditions in our laboratory (see Supplementary Information).

Gene expression profiling and analysis

All gene expression measurements were done at least in triplicate. Samples were stabilized by using RNAProtect bacterial reagent (Qiagen), and total RNA was isolated from exponentially growing cells using a RNeasy mini kit (Qiagen) in accordance with the manufacturer's protocols (see http://www1.qiagen.com). The RNA (10 μg) was then used as the template for complementary DNA synthesis, the product of which was fragmented, labelled and hybridized to an *E. coli* Antisense Genome Array (Affymetrix), which was washed and scanned to obtain an image in accordance with the manufacturer's protocols (see http://www.affymetrix.com). The image files were processed and expression values were normalized using dChip software[29]. We used quantitative real-time polymerase chain reaction with reverse transcription (RT–PCR) to validate expression changes for selected genes. The statistical significance of expression changes for each gene and each strain between aerobic and anaerobic conditions was determined by a *t*-test (log-transformed data, equal variance).

For each deletion strain, we used a two-way ANOVA (strain as the first factor and aerobic or anaerobic condition as the second factor) to determine whether the differential expression observed in the wild-type strain was significantly altered in the deletion strain by determining the statistical significance of the strain–condition interaction effect. For both the *t*-test and the ANOVA analysis, correction for multiple testing was done by using the Benjamini–Hochberg false discovery rate procedure[30], which determines the *P*-value cut-off for each test separately by estimating the FDR resulting from using a particular *P*-value cut-off. The false discovery rate refers to the fraction of true null tests out of all the tests called significant and an FDR of 5% was used for all tests. All gene expression data and the relevant information (such as the MIAME checklist) are provided in the Supplementary Information.

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Competing interests statement The authors declare competing financial interests: details accompany the paper on **www.nature.com/nature**

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Phagocytosis and coniodicidal assays

Alveolar macrophages were exposed to conidia at a macrophage:conidia ratio of 1:5 for 2 h, and 10:1 for 4 h, in the absence or presence of 20 μg ml^{-1} Ptx3 (0.44 × 10^{-6} M Ptx3 protomer) before being evaluated for internalization or coniodicidal activity, respectively (Supplementary information).

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Escherichia coli K-12 undergoes adaptive evolution to achieve *in silico* predicted optimal growth

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Annotated genome sequences[1,2] can be used to reconstruct whole-cell metabolic networks[3–6]. These metabolic networks can be modelled and analysed (computed) to study complex biological functions[7–11]. In particular, constraints-based *in silico* models[12] have been used to calculate optimal growth rates on common carbon substrates, and the results were found to be consistent with experimental data under many but not all conditions[13,14]. Optimal biological functions are acquired through an evolutionary process. Thus, incorrect predictions of *in silico* models based on optimal performance criteria may be due to incomplete adaptive evolution under the conditions examined. *Escherichia coli* K-12 MG1655 grows sub-optimally on glycerol as the sole carbon source. Here we show that when placed under growth selection pressure, the growth rate of *E. coli* on glycerol reproducibly evolved over 40 days, or about 700 generations, from a sub-optimal value to the optimal growth rate predicted from a whole-cell *in silico* model. These results open the possibility of using adaptive evolution of entire metabolic networks to realize metabolic states that have been determined a priori based on *in silico* analysis.

Predictive whole-cell metabolic models can be developed using a constraints-based modelling procedure[15–18]. As an alternative to detailed theory-based models, constraints-based models use the successive imposition of governing constraints (such as mass conservation, thermodynamics, capacity and nutritional environment) to eliminate network functions that exceed the governing constraints. Mathematically this procedure defines a solution space containing all possible metabolic network functions that satisfy the governing constraints. Each particular solution in this space corresponds to a particular state of the metabolic network and therefore a potential behaviour of the cell. Within the solution space defined by the governing constraints, the optimal use of the metabolic network to support growth can be found among all possible solutions using linear optimization[16–19]. However, a single optimal growth condition is of limited interest and a phenotype phase plane (PPP) analysis has been developed to obtain a broad understanding of a metabolic network's optimal properties[20,21]. The PPP analysis evaluates the optimal properties of a metabolic network under a range of environmental conditions (see Methods) and has been used to show that the growth of *E. coli* is consistent with the optimal use of its metabolic network under several defined growth conditions[13,14].

It is not known whether optimal growth is observed on all substrates, and if not, whether adaptive evolution towards optimal growth can be achieved. Furthermore, if such adaptive evolution towards the optimal behaviour occurs, does the endpoint correspond with a priori calculations? To address these issues, we examined prolonged exponential growth of *E. coli* K-12 on several substrates (acetate, succinate, malate, glucose and glycerol). All calculations presented here were made with a previously formulated large-scale *E. coli* metabolic model[12,14], and the model was not adjusted or 'fitted' to the data described.

Batch growth experiments were done using malate as the sole

carbon source with a range of substrate concentrations (0.25–3 g l^{-1}) and temperatures (29–37 °C) to vary the malate uptake rate (MUR). The MUR, oxygen uptake rate (OUR) and growth rate were measured. The measured MUR and OUR data were optimal, as defined by the line of optimality (LO) in the PPP (Fig. 1a). The optimal growth rate of *E. coli* was calculated for all combinations of the MUR and OUR and displayed as a surface over the PPP (Fig. 1b). The experimentally determined growth rates were on the edge of the colour-coded solution space that corresponds to the LO (Fig. 1b). Hence the optimal growth performance of *E. coli* K-12 on malate was predicted *a priori* by using PPP. The results for growth with malate as the sole carbon source were in agreement with previous observations of *E. coli* metabolism for growth on succinate or acetate[14].

A natural question arises: is the optimal performance on malate stable over prolonged periods of time? To address this question, adaptive evolution of *E. coli* on malate was studied for 500 generations. The adaptation resulted in a 19% increase in growth rate. However, the MUR and OUR also increased and maintained metabolic operation on the LO (Fig. 1). Similar adaptive evolution experiments on acetate and succinate resulted in an increased growth rate (20% and 17%, respectively) (Fig. 2). Both the oxygen and substrate uptake rates increased concomitantly to maintain optimal growth as defined and predicted by the PPP analysis.

The growth rate of *E. coli* using glucose as the carbon source was also increased by prolonged exponential growth (Figs 2 and 3). Before adaptive evolution on glucose the cellular growth rate, OUR, and glucose uptake rate (GUR) were experimentally determined over a range of glucose concentrations and temperatures. The experimentally determined values for the GUR and OUR corresponded to points on the LO or slightly in phase 2 (the acetate overflow region) of the PPP (ref. 21) (Fig. 3a). The predicted acetate secretion in phase 2 was experimentally observed and the measured growth rates were on the surface of the solution space near the edge corresponding to the LO (data not shown). *E. coli* was subsequently kept in sustained exponential growth over 500 generations (Fig. 3b, c). The growth rate increased by 17%, as shown by movement of the experimental data points within phase 2 on the surface towards higher growth rates. Thus, as with malate, succinate and acetate, the growth rate of *E. coli* with glucose as the carbon source could be slightly increased with the substrate and oxygen uptake rates moving in phase 2 with some acetate overflow. It was also noted that evolutionary adaptation maintained metabolic operation on the surface of the three-dimensional PPP, as predicted by the physicochemical constraints on the metabolic network. The metabolic operation in the phase 2 provided an increased growth rate with a reduced yield (relative to the LO).

We determined the growth performance over a range of glycerol concentrations and temperature. Unlike growth on malate or glucose, the experimental data points were scattered throughout phase 1 far from the LO (Fig. 4b), indicating sub-optimal growth of

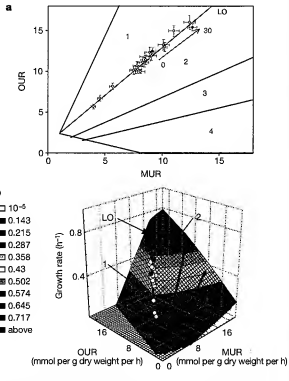

Figure 1 Growth of *E. coli* K-12 on malate. **a**, The malate–oxygen phenotype phase plane (PPP) Phase 1 is characterized by metabolic futile cycles, whereas phase 2 is characterized by acetate overflow metabolism. The line of optimality (LO, in red) separates phases 1 and 2 (ref. 21). Data points (open circles) represent malate concentrations ranging from 0.25–3 g l^{-1}; and temperatures ranging from 29–37 °C. The two data points in blue represent the starting point (day 0) and endpoint (day 30) of adaptive evolution respectively, at a malate concentration of 2 g l^{-1} and a temperature of 37 °C. These data points represent a span of 500 generations. **b**, Three-dimensional representation of growth rates. The *x* and *y* axes represent the same variables as in **a**. The *z* axis represents the cellular growth rate (h^{-1}). OUR, oxygen uptake rate; MUR, malate uptake rate.

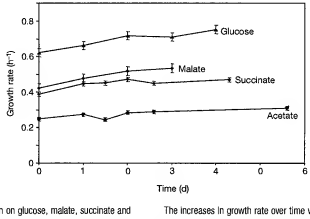

Figure 2 Growth rate during adaptive evolution on glucose, malate, succinate and acetate. Growth conditions were kept constant at a temperature of 37 °C and a substrate concentration of 2 g l^{-1}. We measured growth rate in the exponential phase of growth. The increases in growth rate over time were as follows: glucose (18%), malate (21%), succinate (17%) and acetate (20%). The number of generations for each adaptive evolution was: glucose (500), malate (500), succinate (1,000) and acetate (700).

wild-type *E. coli* K-12 on glycerol, consistent with previous observations[32].

We studied *E. coli* adaptive growth using glycerol as the sole carbon source (2 g l^{-1}), by serial transfer, at a temperature of 30 °C, and with sufficient oxygenation. Growth rate, glycerol uptake rate (G1-UR) and OUR were measured every ten days. Over a 40-day period an evolutionary path (E1) was observed (Fig. 4c). Phenotypic changes were traced in phase 1, eventually converging towards the LO. During this 40-day period, the growth rate more than doubled from 0.23 h^{-1} to 0.55 h^{-1} (Fig. 4a). The substrate uptake and growth rate data obtained under various growth conditions after adaptive evolution were near the LO (Fig. 4d). The evolved strain attained near-optimal growth on glycerol as defined by the *in silico* predictions. A second, independent adaptation experiment gave a similar but non-identical evolutionary trajectory (E2), converging near the same endpoint (Fig. 4c). Finally, a third independent adaptation experiment (E3) was done with a different initial starting point within phase 1. E3 was done at 37 °C and a glycerol concentration of 2 g l^{-1}. The adaptation of *E. coli* to growth on glycerol at 37 °C resulted in motion towards the LO and the growth rate increased by about 30% (Fig. 4a). The final growth rate of the E3 strain was consistent with the *in silico* predictions with respect to the G1-UR, OUR and the growth rate.

To assess the stability of the endpoint of the adaptive evolution, we extended the cultivation on glycerol for an additional 300 generations, or 20 days for the E1 and E2 strains. The data indicated no further change in growth (Fig. 4a). On the sixtieth day of the experiment the E1 and E2 strains exhibited growth on the LO under various growth conditions, reaffirming optimal growth behaviour and the stability of the phenotype (Fig. 4e).

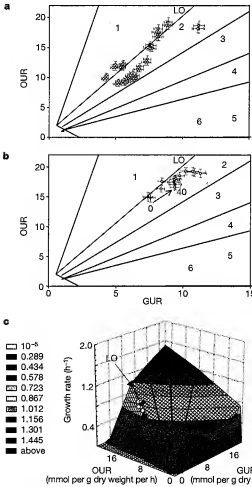

Figure 3 Growth of *E. coli* K-12 on glucose. **a**, The glucose–oxygen PPP. Like the malate–oxygen PPP, phase 1 represents sub-optimal growth and phase 2 is characterized by acetate overflow metabolism. The LO is shown in red. **b**, GUR plotted against OUR along with experimental values for adaptive evolution experiments. Open circles represent measurements during the adaptive evolutionary process, whereas blue circles indicate the beginning and end of evolution. **c**, Three-dimensional rendering of computed growth rate and the experimental data (from **a** and **b**). The *x* and *y* axes represent the GUR and OUR. The *z* axis represents the cellular growth rate (h^{-1}).

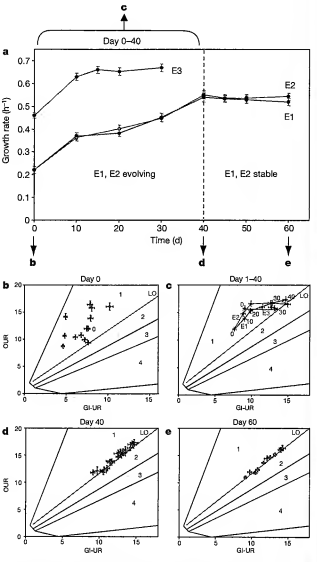

Figure 4 Growth of *E. coli* K-12 on glycerol. **a**, Change in growth rate with time for three adaptive evolution experiments: trajectories E1, E2, and E3. E1 and E2 were performed at 30 °C and E3 at 37 °C. The glycerol concentration was kept constant at 2 g l^{-1} during E1, E2 and E3. **b**, The PPP pre-evolution. The LO is shown in red. The range of glycerol concentrations used was 0.25–2 g l^{-1}. **c**, The PPP during adaptive evolution. Experimental values for E1 are indicated in blue, and for E2 they are indicated in green, and for E3 in red. The starting point of evolution for E1 and E2 is indicated in black (day 0). **d**, The PPP after 40 days (about 700 generations) of evolution. The range of glycerol concentrations used was 0.25–2 g l^{-1}. **e**, The PPP after 60 days (1,000 generations) of evolution. The range of glycerol concentrations used was 0.25–2 g l^{-1}. Data points were obtained using the E1 (blue) and E2 (green) strains. GI-UR, glycerol uptake rate.

Selection pressure is expected to result in optimal performance through an evolutionary process. Optimal growth of *E. coli* on acetate, succinate, malate and glucose is consistent with the predictions of whole-cell *in silico* models. The strain used here has presumably never had to compete for survival using glycerol as the sole carbon source and thus initially utilized this carbon source non-optimally. However, adaptive evolution on glycerol resulted in the a priori calculated optimal growth that was based on the constraints placed on the *E. coli* metabolic network. The adaptive evolutionary process had a reproducible and predictable endpoint.

This study opens up several possibilities. First, it may now be possible to specify optimal network properties *in silico* and achieve them through an adaptive evolutionary process or in combination with a series of other methodologies. *In silico* design of microorganisms could be used to improve their metabolic abilities, production efficiency and/or operational longevity. Second, changes in mRNA expression levels and DNA sequences can now be monitored as cells progress along a defined evolutionary path. Such experiments may yield valuable insight into the molecular design of complex control circuits and their adaptation during evolution. The combination of *in silico* and experimental biology introduced here may make a new series of biological designs attainable.

Constraint-based computational models use an optimization-based procedure to predict cellular states. It is assumed that this optimal state, within the governing constraints, is found by altering the numerical values of the kinetic and regulatory constants through a 'trial-and-error' process. This feature of constraint-based models is a significant departure from other types of mathematical models of cell function, where these parameters are treated as being time-invariant. Thus constraint-based models directly account for the fundamental nature of adaptive evolution. The adaptive evolutionary path itself cannot be predicted; however, the final outcome can be. □

Methods

Strains and media

The *E. coli* K-12 MG1655 annotated genome sequence and the biochemical literature were used to construct the *in silico E. coli* strain[1,2,5], We simulated the metabolic capabilities as previously described with the objective of maximizing growth[11,12,21]. Both growth and maintenance requirements were imposed on the *in silico* model[10–14]. The growth experiments were done in M9 minimal medium with the addition of the carbon source. The growth rate was varied by changing the concentration of the carbon source (ranging from 0.25 to 3 $g l^{-1}$) and the temperature (ranging from 29 °C to 37 °C). *E. coli* MG1655 (ATCC #47076) was used for all of the experiments.

Batch cultures were set up at two different volume scales. One-litre (large) cultures were performed in 1.5-l Erlenmeyer flasks sparging with air. The large-volume batch cultures were used to continuously monitor the oxygen uptake rate (OUR) online with an off-gas analyser. Small (100–250 ml) cultures were grown in 500–1,000 ml Erlenmeyer flasks. For the small-scale cultures the OUR was monitored online polarographically and by measuring the mass transfer coefficient for oxygen (k_La) (see below). The temperature was controlled by using a circulating water bath (Haake). We measured and analysed data during exponential growth. The biomass and the concentration of the substrate (malate, glucose, glycerol) in the media were monitored throughout the experiment.

Analytical procedures

Cellular growth rate was monitored by measuring the absorbance (A, or optical density) at 600 nm and 420 nm and by cell counts (Coulter Electronics). The doubling time was calculated from the growth rate: $t_d = \ln(2)/\mu$. Absorbance to cellular dry weight correlations were determined by two different measurements: (1) spun-down cells were dried at 75 °C to a constant weight; and (2) 25–50 ml samples (taken throughout the culture) were filtered, washed and dried to a constant weight. The concentration of metabolites in the culture media was determined by high-performance liquid chromatography (HPLC) (Rainin Instruments). An aminex HPX-87H ion exchange carbohydrate–organic acid column (Bio-Rad Laboratories) (65 °C) was used with degassed 5 mM sulphuric acid as the mobile phase and ultraviolet detection. Glucose and glycerol were monitored by enzymatic assay (Sigma). The dissolved oxygen in the culture was monitored with a polarographic dissolved oxygen probe (Cole-Parmer Instruments). Oxygen consumption was measured in three different ways: (1) passing the effluent gas through a Servomex oxygen analyser (model 1140C) (Servomex); (2) calculated from the dissolved oxygen reading and k_La measurements; and (3) in a respirometer chamber in a separate 70-ml flask. All three methods used for measuring the OUR gave similar and reproducible results.

Adaptive evolution

Cultures in prolonged exponential growth were started from individual colonies and were grown in micro-carrier spinner flasks at 250 ml within a temperature-controlled incubator. Serial transfers were made during the exponential phase of growth at mid-log-phase ($A_{420\,nm} \approx 0.55$) using an adjusted inoculum volume based on the growth rate of the culture. On a daily basis, the growth rate, time of inoculation, $A_{600\,nm}$ of the culture, and any visual changes in the composition of the culture were recorded. Samples of cultures were stored on a daily basis. The culture was tested weekly for pH and phenotyped (by plating) for any signs of coexistence with a distinct population of mutants or foreign contamination. No discernible differences in colony morphology or signs of foreign contamination were observed during these experiments.

Phenotype phase plane analysis

First, the metabolic reconstruction was done using biochemistry, genomic and physiological data[1–5]. Second, mass balance, capacity and thermodynamic constraints were imposed on the network to define a solution *space*[15]. Third, the best use of the metabolic network for a given objective was identified using linear optimization[16–19]. Fourth, all optimal solutions as a function of two constraints were presented on the PPP[21]. The PPP has distinct phases. Each phase corresponds to a particular type of an optimal solution, which in turn represents a particular flux distribution through the network. Each phase has certain metabolic characteristics; for instance, phase 2 in the figures presented here was characterized by an acetate overflow. The lines that demarcate the phases were defined by changes in the shadow price structure of the optimal solution. The properties of the PPP have been detailed elsewhere[21]. In this study, the optimal utilization of the metabolic network was predicted *a priori*, based on a previously developed *in silico* model[1,2,5].

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12. Edwards, J. S. & Palsson, B. O. The *Escherichia coli* MG1655 *in silico* metabolic genotype: its definition, characteristics, and capabilities. *Proc. Natl Acad. Sci. USA* **97,** 5528–5533 (2000).
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14. Edwards, J. S., Ibarra, R. U. & Palsson, B. O. *In silico* predictions of *Escherichia coli* metabolic capabilities are consistent with experimental data. *Nature Biotechnol.* **19,** 125–130 (2001).
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Competing interests statement The authors declare competing financial interests: details accompany the paper on *Nature*'s website (http://www.nature.com/nature).

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Docket No.: 066662-0092 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	Customer Number: 41552
Palsson, Bernhard	:	Confirmation Number: 1729
Application No.: 09/923,870	:	Group Art Unit: 1631
Filed: August 06, 2001	:	Examiner: Negin, Russell Scott

For: METHODS FOR IDENTIFYING DRUG TARGETS BASED ON GENOMIC SEQUENCE DATA

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jens B. Nielsen, declare as follows:

1. I am a Professor in Systems Biology at Chalmers University of Technology in Götenborg, Sweden. I previously was a full professor at the Technical University of Denmark and served as both the deputy director and the director of Center for Process Biotechnology. I am founder of the Center for Microbial Biotechnology and served as its director until 2007.

2. I earned a Masters of Science in chemical engineering and a Ph.D in biotechnology from the Technical University of Denmark. I did a post doctoral fellowship at the University of Hannover in Germany. I have authored or co-authored more than 270 papers in international peer review journals and am the co-author of three text books in the field of bioreaction engineering, metabolic engineering and metabolomics. I am an inventor or co-inventor on more than 25 issued patents and I am a founder of four companies. I also am a member of Genomatica's Scientific Advisory Board. I am a recipient of a number of prestigious awards, including the Villum Kann Rasmussen's Årslegat and the Merck Award for Metabolic Engineering. A copy of my curriculum vitae and a list of publications is attached as Exhibit 1.

3. I am very familiar with stoichiometric models of metabolism and have read U.S. application serial no. 09/923,870, by Palsson. I also am very familiar with Dr. Palsson's work, including the publication that is the basis of this application (Edwards and Palsson, *Proc. Natl. Acad. Sci. U.S.A.*, 97:5528-33 (2000)). I understand that the invention described in this application is directed to constructing genome specific stoichiometric matrices that can be utilized with flux balance analysis for modeling metabolism. The application claims, in part, a method of simulating a metabolic capability by incorporating metabolic reactions through the use of genome information. I also understand that the claimed invention stands rejected for obviousness over the combination of references to Pramanik and Keasling., *Biotech. and Bioengineering* 56:398-421 (1997) in view of Blattner et al., *Science* 277:1453-69 (1997) and in view of Kunst et al., *Rev. in Microbiol.* 142:905-12 (1991).

4. I know from personal knowledge that at the time Professor Palsson's invention was made respected and prestigious scientists in the field of metabolic engineering publically criticized Professor Palsson's invention and had a strong disbelief that it worked.

5. In July of 1999 I chaired a meeting session at the Biochemical Engineering XI meeting held in Salt Lake City, Utah. I also was a member of this meeting's Advisory Committee. Attached as Exhibit 2 is a copy of the final program for that meeting entitled Biochemical Engineering XI: Molecular Diversity in Discovery and Bioprocessing.

6. During the meeting session that I chaired, Professor Palsson was giving a lecture on the use of metabolic models for simulations of growth and analysis of deletion mutants and other characteristics using the concepts of flux balance analysis. After Professor Palsson's lecture, Professor Jay Bailey from the auditorium requested whether he could provide some comments to the lecture. I agreed as I assumed that it was the normal type of questions and comments that are asked of lecturers at conferences, and considering Professor Baileys prominent position in the field of biochemical engineering and metabolic engineering I expected a good discussion. In fact Professor Bailey has published himself in the field of metabolic flux analysis, and had been working quite a lot on metabolic models.

7. As it turned out, Professor Bailey had prepared 4-5 overhead slides analyzing and criticizing the approach taken by Professor Palsson. It was clear to me that there must have been

some earlier correspondence, and it also seemed like Professor Bailey had prepared "opposition" to another lecture than the one Professor Palsson had just given, as several of the critical points raised by Professor Bailey were in fact addressed in the lecture of Professor Palsson.

8. The main criticism raised by Professor Bailey was that it was not possible to predict metabolic functions and cellular physiology by simply using constraint based simulations. He argued that due to the large degrees of freedom the predictions are not likely to represent true phenotypes. It is correct that there is a large degrees of freedom in the system and that constraint based simulations cannot predict correctly all the fluxes in the network, but correct phenotypes have been demonstrated in numerous examples later to be captured very well by this kind of simulation. The "opposition" from Professor Bailey was supported by several others in the auditorium, so it was quite clear that there was a general belief that the concept would not work.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Jens B. Nielsen

17/6/09

Date

SDO 140831-1.066662.0092

Curriculum vitae

Jens Nielsen

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General information

Date of Birth:	November 17, 1962 in Horsens, Denmark
Citizinship:	Danish
Home Address:	Nedre Fogelbergsgatan 5, SE-411 28 Gothenburg, Sweden

Education and degrees

1986	M.Sc. in Chemical Engineering, Dept. of Biotechnology, DTU, Denmark
1989	Ph.D. in Biochemical Engineering, DTU, Denmark
1995	dr.techn., DTU, Denmark

Employments

1986	**Research Assistant**, School of Engineering, University of Western Ontario, Canada
1987-1989	**PhD student**, Department of Biotechnology, DTU, Denmark
1989	**Post doc**, Institut für Technische Chemie, Universität Hannover, Germany
1990-1995	**Associate Research Professor**, Department of Biotechnology, DTU, Denmark
1995-1996	**Visiting Professor**, Department of Chemical Engineering, MIT, USA
1996-1998	**Associate Professor**, Department of Biotechnology, DTU, Denmark
1998-2008	**Professor**, BioCentrum, DTU, Denmark
2005-	**Adjunct Professor**, Department of Biotechnology, NTNU, Norway
2008-	**Professor**, Department of Chemical and Biological Engineering, Chalmers

Academic Experience

1994-1999	**Founding Chairman**, Danish Biotechnological Forum, Denmark
1995-2000	**Deputy Director**, Center for Process Biotechnology, DTU, Denmark
1995-1998	**Co-ordinator**, Physiological Engineering, Nordic Industrial Fund
1998-2002	**Co-ordinator**, NordPhys, Nordic Industrial Fund
2001-2003	**Director**, Center for Process Biotechnology, DTU, Denmark
2001-2003	**Board Member**, Center for BioProcess Technology, KTH, Sweden
2002-2007	**SAB Member**, Kluywer Center for Genomics of Industrial Fermentations, TU Delft, The Netherlands
2002-2008	**SAB Member**, Max-Planck Institute for Dynamic of Complex Technical Systems, Germany
2004-2007	**Founding Director**, Center for Microbial Biotechnology, DTU, Denmark
2004-2007	**Member of Integrative and Systems Biology Panel**, BBSRC, UK (50 million GPD)
2004-	**Advisory Board**, Society for Biological Engineering, USA
2005	**Chairman of Evaluation Committee**, HepatoSys, Germany
2005-2008	**Co-ordinator**, Yeast Systems Biology Network, EU Framework VI
2006-2007	**Founder and Member**, Danish Biotechnological Society, Denmark
2008-	**Member of Review Panel of SystemsX.ch**, Switzerland (100 million CHF)
2008-	**Co-ordinator**, Systems Biology as a Driver for Industrial Biotechnology, EU Framework VII

Society Memberships

1986-	**The Danish Society for Engineers**, Denmark
1996-	**Member of American Association for the Advancement of Science**, USA
1997-	**Member of the Academy of Technical Sciences**, Denmark
2001-	**Society for Industrial Microbiology**, USA
2004-	**American Chemical Society**, USA
2004-	**Society for Biological Engineering**, USA
2006-	**American Society for Microbiology**, USA

Publications, Patents & Presentations

284	**Peer Reviewed Papers**
20	**Book Contributions**
3	**Text books**: Bioreaction Engineering (1994,2003) (English & Chineese), Metabolic Engineering (1998) (English, Chineese & Japaneese), Metabolome Analysis (2007)
1	**Monograph**
3	**Edited books**
28	**Issue patents (12 patent families)**
>95	**Invited oral presentations at international conferences**
>45	**Invited seminars at universities and companies**

Key Grants

1993-1996	**Nordic Project on Physiological Engineering**, Nordic Industrial Fund (>20 million DKK)
1995-2002	**4 Projects**, EU Framework VI (about 8 million DKK)
1996-1999	**Center for Process Biotechnology**, Danish Technical Research Council (20 million DKK)
1997-2000	**Nordic network NordPhys**, Nordic Industrial Fund (>4 million DKK)
1999-2003	**DABIC**, Danish Technical Research Council (18 million DKK)
2000-2007	**6 Projects**, EU Framework V (about 15 million DKK)
2003-2007	**Heterologous production of polyketides**, Danish Technical Research Council (2.8 million DKK)
2004-2008	**Center for Microbial Biotechnology**, Danish Technical Research Council (36 million DKK)
2005-2009	**3 Projects**, EU Framework VI (about 8.5 million DKK)
2005-2008	**Genome sequencing of *A. balhamycina***, Lundbech Foundation (1.2 million DKK)
2005-2008	**Yeast Systems Biology Network**, EU Framework VI (1.3 million €)
2006-2008	**Yeast in No Gravity**, European Space Agency (2.7 million DKK)
2007-2009	**Systems Biology: From model organisms to application**, NORDFORSK (0.8 million €)
2008-2011	**Systems Biology as a Driver for Industrial Biotechnolog**, EU Framework VII (1 million €)
2008-2013	**UNICELLSYS**, EU Framework VII (5.4 million SEK)

Awards

1989	**Direktør Gorm Petersen's Mindelegat**, Denmark
1994	**Ulrik Brinch og Hustru Marie Brinch's legat**, Denmark
1995	**Fullbright Fellow**, USA
1996	**STVFs Jubilæumspris**, Denmark
2001	**Aksel Tovborg Jensens Legat**/Bjerrum-Brøndsted-Lang Lecture, Denmark
2002	**Villum Kann Rasmussen's Årslegat**, Denmark

2002	**Sunner Memorial Lecture**, Lund University, Sweden
2004	**Merck Award for Metabolic Engineering**, USA
2004	**Hough Memorial Lecture**, Birmingham University, UK

Editorial Activies

1996	**Chemical Engineering Science**, Guest editor of special volume
1999-	**Metabolic Engineering**, Editorial Board (1999-2002), Associate Editor (2003-)
1999-	**Applied Microbiology and Biotechnology**, Editorial Board
2000-	**Bioprocess and Biosystems Engineering**, Associate Editor (2000-2007), Editorial Board (2007-)
2001-	**Biotechnology and Bioengineering**, Associate Editor
2001-	**FEMS Yeast Research**, Editorial Board (2001-2007), Associate Editor (2007-)
2004-	**Journal of Industrial Microbiology and Biotechnology**, Editorial Board
2004-	**Advances in Biochemical Engineering/Biotechnology**, Editorial Board

Teaching Experience

Has organized more than 15 advanced courses on metabolic engineering and systems biology in Denmark, Sweden, Thailand, China and Chile

Has been teaching in several different courses at all levels in the area of biotechnology, fermentation technology, bioreaction engineering, metabolic engineering and systems biology.

Have extensive experience with setting up, organizing and running new courses.

Have been actively involved in the design of new teaching programs at both the BSc and MSc level in the field of biotechnology and systems biology.

Mentoring Experience

Main supervisor of graduated PhD students	43
Co-supervisor of graduated PhD students	22
Current main supervisor of PhD students	12
Current co-supervisor of PhD students	2
Former affiliated post docs	34
Current affiliated post docs	8

Business Experience

1996-	**Consultant** for several internationally leading biotech and pharmaceutical companies
1997	**GeneCare ApS**, Denmark, Founder
1997-2001	**N&N Biotechn ApS**, Denmark, Founder and CEO
2001-2004	**Symbion Venture Capital**, Denmark, Member of Scientific Advisory Board
2002-	**Fluxome Sciences A/S**, Denmark, Founder, CEO (2002-2004), CSO (2002-2008), Member of BOD, Chairman of SAB (2008-) (has raised more than 7 million € in capital)
2002-2007	**Gothia Yeast Solutions**, Sweden, Member of SAB
2006-	**MycoTeQ A/S**, Denmark, Founder and Chairman of BOD (has raised about 1 million € in capital)
2008-	**GlycoFi, Inc.**, USA, Member of SAB
2008-	**Promethegen, Inc.**, USA, Member of SAB

Organization of Conferences

2000-	Member of organizing and scientific committee of more than 25 conferences
2000	**ESBES3**, Denmark, Chairman (~300 delegates)
2002	**Analysis of Microbial Cells at the Single Cell Level**, Denmark, Co-Chair (~100 delegates)
2002	**Metabolic Engineering IV**, Italy, Chairman (~250 delegates)
2004	**European Conference on Fungal Genetics VI**, Denmark, Co-Chair (~800 delegates)
2005	**ECB12**, Denmark, Chairman of Scientific Committee (~1200 delegates)

List of Publications for Jens Nielsen

Research papers in international journals with peer review

1988

(1) K. Nikolajsen; **J. Nielsen**; J. Villadsen (1988)
In-line flow injection analysis for monitoring lactic acid fermentations. *Anal. Chim. Acta* **214**:137-145

1989

(2) **J. Nielsen**; K. Nikolajsen; J. Villadsen (1989)
FIA for on-line monitoring of important lactic acid fermentation *variables. Biotechnol. Bioeng.* **33**:1127-1134

(3) **J. Nielsen**; C. Emborg; K. Halberg; J. Villadsen (1989)
Compartment model concept used in the design of fermentation with recombinant microorganisms *Biotechnol. Bioeng.* **34**:478-486

1990

(4) **J. Nielsen**; K. Nikolajsen; S. Benthin; J. Villadsen (1990)
Application of flow-injection analysis in the on-line monitoring of sugars, lactic acid, protein, and biomass during lactic acid fermentations. *Anal. Chim. Acta* **237**:165-175

(5) G. Wehnert; K.-D. Anders; C. Bittner; R. Kammeyer; U. Hübner; **J. Nielsen**; T. Scheper (1990)
Ein kombinierter Fluoreszenz-/Streulichtsensor und dessen Einsatz zur Prozessbeobachtung in der Biotechnologie. *Chem.-Ing.-Tech.* **62**:211-212

1991

(6) S. Benthin; **J. Nielsen**; J. Villadsen (1991)
A simple and reliable method for the determination of cellular RNA content. *Biotechnol. Techn.*. **5**:39-42

(7) **J. Nielsen**; K. Nikolajsen; J. Villadsen (1991)
Structured modelling of a microbial system 1. A theoretical study of the lactic acid fermentation. *Biotechnol. Bioeng.* **38**:1-10

(8) **J. Nielsen**; K. Nikolajsen; J. Villadsen (1991)
Structured modelling of a microbial system 2. Experimental verification of a structured lactic acid fermentation model. *Biotechnol. Bioeng.* **38**:11-23

(9) K. Nikolajsen; **J. Nielsen**; J. Villadsen (1991)
Structured modelling of a microbial system 3. Growth on mixed substrates. *Biotechnol. Bioeng.* **38**:24-29

(10) **J. Nielsen**; A. G. Pedersen; K. Strudsholm; J. Villadsen (1991)
Modelling fermentations with recombinant microorganisms : Formulation of a structured model. *Biotechnol. Bioeng.* **37**:802-808

(11) S. Benthin; **J. Nielsen**; J. Villadsen (1991)
Characterisation and application of precise and robust flow injection analyzers for on-line measurement during fermentations. *Anal. Chim. Acta* **247**:45-50

(12) L. H. Christensen; **J. Nielsen**; J. Villadsen (1991)
Monitoring of substrates and products during fed-batch penicillin fermentations on complex media. *Anal. Chim. Acta* **249**:123-136

(13) L. H. Christensen; **J. Nielsen**; J. Villadsen (1991)
Delay and dispersion in an in-situ membrane probe for bioreactors. *Chem. Eng. Sci.* **46**:3304-3307

1992

(14) S. Benthin; **J. Nielsen**; J. Villadsen (1992)
Flow Injection Analysis of micromolar concentrations of glucose and lactate in fermentation media. *Anal. Chim. Acta* **261**:145-153

(15) S. Benthin; **J. Nielsen**; J. Villadsen (1992)
Anomeric specificity of glucose uptake systems in *Lactococcus cremoris*, *Escherichia coli* and *Saccharomyces cerevisiae:* Mechanisms, kinetics and implications. *Biotechnol. Bioeng.* **40**:137-146

(16) **J. Nielsen** (1992)
On-line monitoring of microbial processes by flow injection analysis. *Proc. Control Qual.* **2**:371-384

(17) C. L. Johansen; L. H. Christensen; J. Villadsen; **J. Nielsen** (1992)
Monitoring and control of fed-batch penicillin fermentation. *Comp. Chem. Eng.* **16**:S297-S304

(18) K. Strudsholm; **J. Nielsen**; C. Emborg (1992)
Product formation during batch fermentation with recombinant *Escherichia coli* containing a runaway plasmid. *Bioprocess Eng.* **8**:173-181

1993

(19) A. G. Pedersen; M. Bundgaard; O. Hassager; **J. Nielsen**; J. Villadsen (1993)
Rheological characterization of media containing *Penicillium chrysogenum*. *Biotechnol. Bioeng.* **41**:162-164

(20) **J. Nielsen** (1993)
A simple morphologically structured model describing the growth of filamentous microorganisms. *Biotechnol. Bioeng.* **41**:715-727

(21) M. Carlsen; L. H. Christensen; **J. Nielsen** (1993)
Flow-injection analysis for measurement of penicillin V in fermentation samples. *Anal. Chim. Acta* **274**:117-123

(22) M. Carlsen; H. Meier; F. Lantreibecq; C. Johansen; R. W. Min; **J. Nielsen** (1993)
On-line monitoring of penicillin V during penicillin fermentations : A comparison of two different methods based on FIA. *Anal. Chim. Acta* **279**:51-58

(23) S. Benthin; **J. Nielsen**; J. Villadsen (1993)
Transport of sugars via two anomer-specific sites on mannose-phosphotransferase system in *Lactococcus cremoris* : In vivo study of mechanism, kinetics and adaption. *Biotechnol. Bioeng.* **42**:440-448

(24) **J. Nielsen** (1993)
Simulation of bioreactions. *Comp. Chem. Eng.* **18**:S615-S620

(25) S. Benthin; **J. Nielsen**; J. Villadsen (1993)
Two uptake systems for fructose in *Lactococcus lactis* subsp. *cremoris* FD1 produce glucolytic and gluconeogenic fructose phosphates and induce oscillations of growth and lactic acid formation. *Appl. Environ. Microbiol.* **59**:3206-3211

1994

(26) S. Benthin; **J. Nielsen**; J. Villadsen (1994)
Galactose expulsion during lactose metabolism in *Lactococcus lactis* subsp. *cremoris* FD1 due to dephosphorylation of intracellular galactose-6-phosphate. *Appl. Environ. Microbiol.* **60**:1254-1259

(27) A. G. Pedersen; M. Bundgaard-Nielsen; **J. Nielsen**; J. Villadsen (1994)
Characterization of mixing in stirred tank bioreactors equipped with Rushton turbines. *Biotechnol. Bioeng.* **44**:1013-1017

(28) A. G. Pedersen; H. Andersen; **J. Nielsen**; J. Villadsen (1994)
A novel technique based on ^{85}Kr for quantification of gas-liquid mass transfer in bioreactors. *Chem.Eng. Sci.* **49**:803-810

(29) S. Benthin; U. Schulze; **J. Nielsen**; J. Villadsen (1994)
Growth energetics of *Lactococcus cremoris* FD1 during energy-, carbon- and nitrogen limitation in steady state and transient cultures. *Chem. Eng. Sci.* **49**:589-610

(30) L. H. Christensen; G. Mandrup; **J. Nielsen**; J. Villadsen (1994)
A robust LC method for studying the penicillin fermentation. *Anal. Chim. Acta* **296**:51-62

(31) L. H. Christensen; **J. Nielsen**; J. Villadsen (1994)
Degradation of penicillin V in fermentation media. *Biotechnol. Bioeng.* **44**:165-169

(32) M. A. Hjortso; **J. Nielsen** (1994)
A conceptual model of autonomous oscillations in microbial cultures. *Chem. Eng. Sci.* **49**:1083-1095

(33) **J. Nielsen**; C. L. Johansen; J. Villadsen (1994)
Culture fluorescence measurements during batch and fed-batch cultivations with *Penicillium chrysogenum. J. Biotechnol* **38**:51-62

(34) M. Carlsen; J. Marcher; **J. Nielsen** (1994)
An improved FIA-system for measuring α-amylase in cultivation *media, Biotechnol. Tech.* **8**:479-482

1995

(35) H. Jørgensen; **J. Nielsen**; J. Villadsen; H. Mølgaard (1995)
Analysis of the penicillin V biosynthesis during fed-batch cultivations with a high yielding strain of *Penicillium chrysogenum, Appl. Microbiol. Biotechnol.* **43**:123-130

(36) **J. Nielsen**; P. Krabben (1995)
Hyphal growth and fragmentation of *P. chrysogenum* in submerged cultures. *Biotechnol. Bioeng.* **46**:588-598

(37) **J. Nielsen**; C. L. Johansen; M. Jacobsen; P. Krabben; J. Villadsen (1995)
Pellet formation and fragmentation in submerged cultures of *Penicillium chrysogenum* and its relation to penicillin production, *Biotechnol. Prog.* **11**:93-98

(38) H. Jørgensen; **J. Nielsen**; J. Villadsen; H. Mølgaard (1995)
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FINAL PROGRAM

BIOCHEMICAL ENGINEERING XI: MOLECULAR DIVERSITY IN DISCOVERY AND BIOPROCESSING

July 25 - 30, 1999
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Poster Session A
Poster Session B

Sunday, July 25, 1999

12:00 noon - 2:30 p.m. Conference Registration (Ballroom Atrium)

2:30 p.m. - 3:00 p.m. **Welcoming Remarks** (Ballroom I)
George Georgiou, University of Texas at Austin
Steven Lee, Merck & Co., Inc.
Allen Laskin, United Engineering Foundation

PLENARY SPEAKERS:

3:00 p.m. - 4:00 p.m. ***Whole-Genome RNA and Protein Regulatory Networks in E-Coli and S. Cerevisiae***
G. Church
Harvard University

4:00 p.m. - 5:00 p.m. ***The Chemical Industry in the 21st Century: Role of Biotechnology***
J. Miller
CTO, DuPont

5:00 p.m. - 5:30 p.m. Break (Ballroom Atrium)

5:30 p.m. - 6:30 p.m. ***Using New Gene Targets in Drug Discovery with Effective Throughput Screening Systems***
P. Fernandes
CEO, Small Molecule Therapeutics

6:30 p.m. - 7:30 p.m. ***Methods for Mining DNA Microarray Data***
G. Stephanopoulos
Department of Chemical Engineering, Massachusetts Institute of Technology, USA

7:30 p.m. - 9:00 p.m. Dinner (Ballroom II & III)

9:00 p.m. - 10:30 p.m. Opening Reception (Ballroom Atrium)

Monday, July 26, 1999

7:00 a.m. - 8:15 a.m. Breakfast (Ballroom II & III)

SESSION (a): EXPLOITING MICROBIAL DIVERSITY (Ballroom I)
Session Chairs: R. Kelly, North Carolina State University
V. Nagarajan, DuPont

8:15 a.m. - 8:20 a.m. **Introduction:** Session Chairs

8:20 a.m. - 9:00 a.m. ***Gene Acquisition in Bacterial Genome Evolution***
J. Roth
University of Utah

9:00 a.m. - 9:30 a.m. ***How Bacteria Talk to Each Other: Quorum Sensing in Escherichia Coli, Salmonella Typhimurium and Vibrio Harveyi***
L. Bassler
Princeton University

9:30 a.m. - 10:00 a.m. ***Predicting the Consequences of Genome Reorganization***
I. Molineux
University of Texas at Austin

10:00 a.m. - 10:30 a.m. Coffee Break (Ballroom Atrium)

10:30 a.m. - 11:00 a.m. ***Hyperthermophilic Genome Sequences: So Many Interesting Enzymes, So Little Time***
R. Kelly
North Carolina State University

11:00 a.m. - 11:30 a.m. ***Purification, Characterization and Process Considerations of Cryophilic Proteases of Marine Origin***
J. Asenjo
University of Chile

11:30 a.m. - 12:00 noon ***Harvesting Biomolecules for the Tree of Life***
E. Mathur
Diversa Corporation

12:00 noon - 12:30 p.m. ***Genetic and Biochemical Diversity of Bacterial in Commercial Wastewater Bioreactors***
V. Nagarajan
DuPont Company

12:30 p.m.- 1:45 p.m. Lunch (Ballroom II & III)

1:45 p.m. - 4:00 p.m. *Ad hoc* Sessions and/or free time

SESSION (b): NOVEL HIGH THROUGHPUT TECHNOLOGIES IN DISCOVERY (Ballroom I)
Session Chairs: G. Georgiou, University of Texas at Austin
J. Chalmers, Ohio State University

4:00 p.m. - 4:05 p.m. **Introduction:** Session Chairs

4:05 p.m. - 4:35 p.m. ***Non-Invasive Monitoring of Pathogens, Tumor Cells and Gene Expression in a Living Animal***
P. Contag
CEO, Xenogen Corp.

4:35 p.m. - 5:05 p.m. ***Directed Enzyme Evolution on Bacterial Surfaces***
B.L. Iverson
University of Texas at Austin

5:05 p.m. - 5:35 p.m. ***Lead Identification from Encoded Synthetic Combinatorial Libraries Via a Self-Selective, Positive Feedback Reporter System***
G. Lalonde
Affymax Research Institute

5:35 p.m. - 6:00 p.m. Coffee Break (Ballroom Atrium)

6:00- p.m. - 6:30 p.m. ***Transcript Profile and Proteomics-Based Views of Biological Models for Pharmaceutical Development***
J. Seilhamer
Incyte Pharmaceuticals, Inc.

6:30 p.m. - 7:00 p.m. ***Microfabricated Chemical Analyses Systems***
Mark Burns
University of Michigan

7:00 p.m. - 7:30 p.m. ***Exploitation of Immunomagnetic Labeling: Rapid Cell Sorting and Fractionation***
J. Chalmers
Ohio-State University, USA

7:30 p.m. - 9:00 p.m. Dinner (Ballroom II & III)

9:15 p.m. - 10:30 p.m. **Poster Session A** and Social Hour (Ballroom Atrium)

Tuesday, July 27, 1999

7:00 a.m. - 8:25 a.m. Breakfast (Ballroom II & III)

SESSION (C) : DISCOVERY AND DESIGN OF MACROMOLECULES (Ballroom I)
Session Chairs: B.L. Iverson, University of Texas at Austin
V. Schellenberger, Genencor International, USA

8:25 a.m. - 8:30 a.m. **Introduction:** Session Chairs

8:30 a.m. - 9:00 a.m. ***Evolution of Ribozymes, Proteins and Pathways***
A. Ellington
University of Texas at Austin

9:00 a.m. - 9:30 a.m. ***Directed Evolution of Subtilisin***
V. Schellenberger
Genencor International, USA

9:30 a.m. - 10:00 a.m. ***Directed Evolution of Protein Recognition. Stability and Expression by Yeast Surface Display***
K.D. Wittrup
University Illinois

10:00 a.m. - 10:30 a.m. Coffee Break (Ballroom Atrium)

10:30 a.m. - 11:00 a.m. ***Creation of New Cytotoxic Antitumor Antibodies by Glycosylation Engineering***
J.E. Bailey
Institute of Biotechnology, ETH Zurich, Switzerland

11:00 a.m. - 11:30 a.m. ***Molecular Breeding of Genes, Pathways and Genomes by DNA Shuffling***
W.P.C. Stemmer
Maxygen, Inc., USA

11:30 a.m. - 12:00 noon Discussion

12:00 noon - 1:30 p.m. Lunch (Ballroom II & III)

1:45 p.m. - 3:00 p.m. *Ad hoc* Sessions and/or free time

SESSION (d): BIOINFORMATICS (Ballroom I)
Session Chairs: J. Nielsen, Technical University Denmark, Denmark
B.O. Palsson, University of California at San Diego

3:15 p.m. - 3:45 p.m. ***Definition of the E. Coli Metabolic Genotype: Basic Concepts, Scientific and Applied Uses***
B.O. Palsson
University of California at San Diego

3:45 p.m. - 4:15 p.m. ***Strategies for Prediction of Orphan Protein Function***
S. Brunak
Technical University of Denmark

4:15 p.m. - 4:45 p.m. ***Pathmap; A New Tool for Visualization of Gene Expression Data***
John Rogers
Parke-Davis Pharmaceutical Research

4:45 p.m. - 5:15 p.m. ***Bioinformatics in the Postgenomic Era***
S. Subramanian
University of Illinois UC

5:15 p.m. - 5:45 p.m. Coffee Break (Ballroom Atrium)

SESSION (e): DISCOVERY AND PRODUCTION OF BIOACTIVE SMALL MOLECULES
Session Chairs: A.E. Barron, Northwestern University
D.S. Clark, University of California - Berkeley

5:45 p.m. - 6:15 p.m. ***Integrating Biocatalysis into the Drug Discovery Pipeline***
D.S. Clark
University of California - Berkeley

6:15 p.m. - 6:45 p.m. ***A Stress Promoter-Based System for Antibiotics Screening***
F. Baneyx
Department of Chemical Engineering, University of Washington, USA

6:45 p.m. - 7:15 p.m. ***The Challenge of Ultra-High Throughput Screen Technology: Implementation of Biological Assays to 3,456 Well Format***
L. Mere
Aurora Biosciences Corporation

7:15 p.m. - 7:45 p.m. ***Peptoids: A High Diversity Family of Synthetic, Protease-Stable Peptide Mimics***
A.E. Barron
Chemical Engineering Department, Northwestern University, USA

7:45 p.m. - 8:00 p.m. Discussion

8:00 p.m. - 9:30 p.m. Dinner (Ballroom II & III)

9:30 p.m. - 10:30 p.m. Social Hour (Ballroom Atrium)

Wednesday, July 28, 1999

7:00 a.m. - 8:15 a.m. Breakfast (Ballroom II & III)

SESSION (f): HIGH THROUGHPUT BIOPROCESING (Ballroom I)
Session Chairs: S. Ozturk, Bayer Corporation
S. Drew, Merck & Co.

8:15 a.m. - 8:20 a.m. **Introduction:** Session Chairs

8:20 a.m. - 8:50 a.m. ***An "Animal" on a Chip: Preclinical Evaluation of Pharmaceuticals***
M. L. Shuler
School of Chemical Engineering
Cornell University, USA

8:50 a.m. - 9:20 a.m. ***An Efficient Integrated Approach to Bioprocess Development***
R. Greasham
Merck & Co.

9:20 a.m. - 9:50 a.m. ***Biocatalytic Systhesis of Chiral Intermediate for Anti-Hypertension Drugs***
R. Patel
Bristol-Myers Squibb Pharmaceutical Research Institute

9:50 a.m. - 10:20 a.m. Coffee Break (Ballroom Atrium)

10:20 a.m. - 10:50 a.m. ***Transient Gene Expression for Biotech Research***
A. Bernard
Serono Pharmaceutical Research Institute, S.A., Switzerland

10:50 a.m. - 11:20 a.m. ***High Throughput Bioprocessing: New Directions in Pharmaceutical Drug Development***
S. Ozturk
Bayer Corporation

11:20 a.m. - 11:50 .a.m. ***Protein Production in Transgenic Plants***
V. Paradkar
Monsanto Company

11:50 a.m. - 12:00 noon Discussion

12:00 noon - 1:30 p.m. Lunch (Ballroom II & III)

1:30 p.m. - 4:00 p.m. **Poster Session B**/*Ad hoc* Sessions and/or free time (Ballroom Atrium)

SESSION (g): CONTROLLING PRODUCT HETEROGENEITY (Ballroom I)
Session Chairs: E.T. Papoutsakis, Northwestern University
M.L. Shuler, Cornell University

4:15 p.m. - 4:20 p.m. **Introduction:** Session Chairs

4:20 p.m. - 4:50 p.m. ***Genetic and Physiological Manipulation of the Protein Glycosylation Pathway***
P. Jenkins
Lilly Research Laboratories, Eli Lilly & Co.

4:50 p.m. - 5:20 p.m. ***Controlling Secretory Processing to Improve Product Quality and Homogeneity***
M.J. Betenbaugh
Department of Chemical Engineering, Johns Hopkins University, USA

5:20 p.m. - 5:50 p.m. ***DNA Microarray for Metabolic Engineering***
J.C. Liao
Deparment of Chemical Engineering, University of California Los Angeles, USA

5:50 p.m. - 6:15 p.m. Coffee Break (Ballroom Atrium)

6:15 p.m. - 6:45 p.m. ***The Domain-Bypass Mechanism for Creation of Structural Diversity in the Pikromycin Biosynthetic Gene Cluster***
D. Sherman
University of Minnesota

6:45 p.m. - 7:15 p.m. ***Sugars to Plastics, then to Fine Chemical Monomers: Production of Various Enantiomerically Pure ®- Hydroxycarboxylic Acids***
S.Y Lee
Chemical Engineering Department, Korea Advanced Institute of Science and Technology, Korea

7:30 p.m. - 10:00 p.m. Conference Banquet/AMGEN Award and Social Hour (Ballroom II & III)
AMGEN Award Lecture
Cell Engineering: Understanding and Controlling Receptor Processes
Douglas A. Lauffenburger
Massachusetts Institute of Technology

Thursday, July 29, 1999

7:00 a.m. - 8:15 a.m. Breakfast (Ballroom II & III)

SESSION (h): DISCOVERY OF BIOLOGICAL MATERIALS (Ballroom I)
Session Chairs: D. Kaplan, Tufts
S.Y. Lee, KAIST, Korea

8:15 a.m. - 8:20 a.m. **Introduction:** Session Chairs

8:20 a.m. - 8:50 a.m. ***Bioengineered Microbial Lipopolysaccharides and Glycolipids***
D. Kaplan

Tufts

8:50 a.m. - 9:20 a.m. ***Bioactive Biomaterials for Engineering Tissue Healing***
J.A. Hubbell
ETH Zürich

9:20 a.m. - 9:50 a.m. ***Molecular Weight Control in Bacterial Hyaluronic Acid Production***
L. Nielsen
Chemical Engineering Department, University of Queensland, Australia

9:50 a.m. - 10:20 a.m. Coffee Break (Ballroom Atrium)

10:20 a.m. - 10:50 a.m. ***S.-Layers: Fundamentals and Applications in Molecular Nanotechnology and Biomimetics***
Prof. U.B. Sleytr
University of Agricultural Sciences, Vienna, Austria

10:50 a.m. - 11:20 a.m. ***Bioinductive Polymer Coatings for Implantable Glucose Biosensors***
J. Koberstein
University of Connecticut

11:20 a.m. - 11:50 a.m. ***Properties and Biotechnological Applications of the Azotobacter Vinelandii Modular Type Mannuronan C-5 Epimerases***
S. Valla
Norwegian University of Science and Technology

11:50 a.m. - 12:00 noon Discussion

12:00 noon - 1:30 p.m. Lunch (Ballroom II & III)

1:30 p.m. - 3:30 p.m. *Ad hoc* Sessions and/or free time

SESSION (i): PRODUCTION OF COMPLEX PRODUCTS (Ballroom I)
Session Chair: J.R. Swartz, Stanford University

3:45 p.m. - 3:50 p.m. ***Cell Free Protein Synthesis for Discovery and Production***
J.R. Swartz
Stanford University

4:20 p.m. - 4:50 p.m. ***Production of Viral Vaccines: A Role For Biochemical Engineering***
D. Robinson
Merck & Co., USA

4:50 p.m. - 5:20 p.m. ***O2 and its Transport in Hematopoietic Life and Death***
E.T. Papoutsakis
Northwestern University, USA

5:20 p.m. - 5:45 p.m. Coffee Break (Ballroom Atrium)

5:45 p.m. - 6:15 p.m. ***Stem Cell-Based Cellular and Tissue Engineering***
P. Zandstra
University of Toronto, Canada

6:15 p.m. - 6:45 p.m. ***The Production of Plasmid DNA for Gene Therapy: The Cell Lysis Step***
A.W. Nienow
Centre for Bioprocess Engineering, School of Chemical Engineering, The University of Birmingham, UK

6:45 p.m. - 7:15 p.m. ***Manipulation of the Glycosylation of Recombinant Proteins Produced in Cho Cells by Overexpression of Glycosyltransferase***
L. Krummen
Genentech, Inc., USA

7:15 p.m. - 7:30 p.m. Discussion

7:30 p.m. - 9:00 p.m. Dinner (Ballroom II & III)

9:00 p.m. - 10:00 p.m. Social Hour (Ballroom Atrium)

Friday, July 30, 1999

7:00 a.m. - 8:30 a.m. Breakfast (Ballroom II & III)

8:30 a.m. - 10:00 a.m. Wrap-up Discussion (Ballroom I)

10:00 a.m. - 12:00 noon Free Time

12:00 noon - 1:30 p.m. Lunch (Ballroom II & III)

1:30 p.m. Conference Adjournment

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Poster Session A

A - 1 ***Cytoplasmic Expression of Disulfide Bonded Proteins in E. Coli***
Paul H. Bessette and George Georgiou
Department of Chemical Engineering, University of Texas at Austin, USA
Xiaoming Zhang
Department of Molecular Biology, University of Texas at Austin, USA

A - 2 ***Secretory Production of Recombinant Proteins Using an Artificial Signal Sequence***
Sang Yup Lee and Jong Hyun Choi
Chemical Engineering Department, Korea Advanced Institute of Science and Technology, Korea

A - 3 ***High Level Production of Human Leptin and Its Purification***

Sang Yup Lee and Ki Jun Jeong
Department of Chemical Engineering, Korea Advanced Institute of Science and Technology, Korea

A - 4 ***Amino Acid Depletion Model and Experimental Validation for Recombinant E. coli***
Kevin J.-R. Clark, Fu'ad Haddadin and Sarah W. Harcum
Department of Chemical Engineering, New Mexico State University, Las Cruces, NM

A - 5 ***Characterisation of Plasmid DNA Production in Escherichia Coli Fermentation for Gene Therapy***
R.D. O'Kennedy and E. Keshavarz-Moore
Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, UK

A - 6 ***Extremophilic Enzymes: Heterologous Gene Expression and Use as Biocatalysts***
Dr. Julian B. Chaudhuri, Dr. Helen Connaris, Gerald Sellek, Prof. Michael Danson and Dr. David Hough
Department of Chemical Engineering, University of Bath, UK

A -7 ***Production of Antifungal Molecules Using Hydrothermal Marine Bacteria***
Joseph Boudrant
Laboratory of Chemical Engineering Sciences-CNRS, France
Joël Coulon and Roger Bonaly
UMR UHP-CNRS, Biochimie Microbienne, Faculté de Pharmacie, France
Georges Barbier
Laboratory of Microbiology, IFREMER, Centre de Brest, France
Jacques Dietrich
Laboratory of Biotechnology, IFREMER, Centre de Brest, France

A - 8 ***Extracellular Proteinases from Psychrotrophs: Keratinase***
Quamrul Hasan and Daniel G. Moran
Procter & Gamble Far East, Inc., Research & Development Division, Japan
Yasutaka Morita and Eiichi Tamiya
Japan Advanced Institute of Science and Technology, The School of Materials Science, Japan

A - 9 ***Study of Dynamic Response to Growth Rate Oscillation in Recombinant Bacillus Subtilis Culture***
S. Chuen-Im
Biotechnology and Biochemical Engineering Group, Department of Food Science and Technology, University of Reading, UK
D.L. Pyle and H.C. Lynch
Department of Food Science & Technology, University of Reading, UK

A - 10 ***Modelling of Baeyer-Villiger Monooxygenase Biocatalytic Reactions***
Matthew Hogan and John Woodley
Department of Biochemical Engineering, University College London, UK

A - 11 ***Axial Flow Up-Pumping Impellers in Industrial Fermentation Processes***
Samun K. Dahod, Ph.D.
Abbott Laboratories, USA

A - 12 ***Is the Production of Biomaterials by Fermentation Economically Viable?***
Anton PJ Middleberg
Department of Chemical Engineering, University of Cambridge, UK
Yao Ling and Richard J. van Wegen
University of Adelaide, Australia

A - 13 ***Mathematical Model of Inorganic Phosphate Controlled Expression of Dengue Envelope Protein by Saccharomyces Cerevisiae***
Anan Tongta
School of Bioresources and Technology, King Mongkut's University of Technology Thonbury, Thailand

Chulee Yompakdee
Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonbury, Thailand

A - 14 ***Production and Recovery of Human Endostatin from Pichia Pastoris***
Joseph Shiloach, Loc Trinh and Santosh Noronha
Biotechnology Unit, LCDB, NIDDK, National Institute of Health, USA

A - 15 ***NADH Oscillations in Brewers Yeast***
Claus Emborg
Center for Process Biotechnology, Department of Biotechnology, Technical University of Denmark, Denmark
Jes Tobiassen
Alfred Jørgensen Lab, Copenhagen

A - 16 ***Exploiting the Diversity in Cell Wall Hydrolases of Trichoderma Reesei***
H.J. Meerman
Genencor International, Inc.

A - 17 ***Cultivation and Direct Regeneration System of Embryogenic Rice Callus Using Macroporous Support***
Hiroyuki Honda, Kwan Hoon Moon, Chunzhao Liu and Takeshi Kobayashi
Department of Biotechnology, Graduate School of Engineering, Nagoya University, Japan

A - 18 ***Glycosylational Engineering in Insect Cells: Effects of Gal T Overexpression***
Michael J. Betenbaugh, Eric Ailor and Y.C. Lee
Department of Chemical Engineering, Johns Hopkins University, USA
Noriko Takahashi
Nakano Vinegar Co. Ltd.
Donald Jarvis
University of Wyoming, USA

A - 19 ***Manipulation of the Apoptosis Pathway in Mammalian Cell Culture***
Tina M. Sauerwald, Bruno Figueroa, Jr., J. Marie Hardwick and Michael J. Betenbaugh
Department of Chemical Engineering, Johns Hopkins University, USA

A - 20 ***Impact of Dolichol Monophosphate Supplementation on Recombinant Gamma-Interferon Glycosylation***
Inn H. Yuk and Prof. Daniel I.C. Wang

Massachusetts Institute of Technology, USA

A - 21 ***Strategies for Production of High Titer Herpes-Based Gene Therapy Vectors***
J.C. Wetchuck, A. Ozuer, B. Russell and M.M. Ataai
Chemical Engineering, University of Pittsburgh, USA
J. Glorioso
Molecular Biology and Biochemistry, University of Pittsburgh, USA

A - 22 ***High Density Culture of Panax Notoginseng Cells in Bioreactors for Saponin Production***
H. Yao and J.J. Zhong
State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, China

A - 23 ***Phase Formation Behavior and Kinetics of the Separation of Phases and Proteins in Aqueous Two-Phase Systems***
B. A. Andrews, E. Huenupi, R. Muñoz, and J. A. Asenjo
Center for Biochemical Engineering and Biotechnology, Department of Chemical Engineering, University of Chile, Santiago, Chile

A - 24 ***Effects of Bone Marrow Architectures on Oxygen Tensions and Gradients Experienced by Hematopoietic Cells in vivo***
Dominic Chow
Northwestern University

A - 25 ***Molecular Analysis of spo0A in Clostridium Acetobutylicum - Progress Towards Decoupling Stationary Phase Phenomena in Solventogenic Bacteriacteria***
Latonia M. Harris
Northwestern University

A - 26 Surface Display of Enzymes as a Selective Screening System
Jae-Gu Pan
Korea Research Institute of Bioscience and Biotechnology

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Poster SessionB

B - 1 ***Engineering Microorganisms for Heavy Metal Removal***
Douglas S. Clark, J.D. Keasling, Clifford L. Wang, Sang-Weon Bang and Andrew C. Magyarosy
Department of Chemical Engineering, University of California-Berkeley, USA

B - 2 ***Application of Flow Cytometry to Study Substrate and Product Toxicity in the Indene Bioconversion***
Ashraf Amanullah
Merck and Co. / University College London, Department of Biochemical Engineering, UK
C.J. Hewitt, A.W. Nienow
School of Chemical Engineering, The University of Birmingham, UK
M. Chartrain, B.C. Buckland and S.W. Drew
Department of Bioprocess Research and Development, Merck Research Laboratories, Merck and Co. Inc., USA

J.M. Woodley
Department of Biochemical Engineering, University College London, UK

B - 3 ***Sedimentation of Manganese/Iron Ore Fines from Wash Water of an Ore Washing Plant***
A.D. Agate
Agharkar Research Institute

B - 4 ***DNA Cleavage and Biological Activities of Substituted Naphthyle Imides***
Dong-Zhi Wei, Dong-Hui Zhu and Jiang-Chao Qian
Research Institute of Biochemistry, State Key Laboratory of Bioreactor Engineering, East China University of Science & Technology, China
Tian-Bao Huang and Xu-Hong Qian
Institute of Pharmaceuticals & Pesticides, East China University of Science & Technology

B - 5 ***Bioconversion in a Membrane Bioreactor-Experimental and Computer Simulated Comparisons***
Preeta Tyagi
Youngsoft, Inc.
Prof. S.N. Upadhyay
Institute of Technology, India

B - 6 ***A Rapid and Rational Method for Selection of Cofactor Requiring Processes***
Katie C. Thomas and John M. Woodley
The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, UK

B - 7 ***Directed Discovery and Analysis of Antisense Oligonucleotides***
Martin L. Yarmush, Charles M. Roth, S. Patrick Walton, and Arul Jayaraman
Center for Engineering in Medicine, Massachusetts General Hospital, USA

B - 8 ***Design and Discovery of Poly-(1-3)-Trans-(2-2)-GPDD from Acholeplasma Laidlawii***
L.L. Matz
Matz & Associates, USA

B - 9 ***Thermostable Peptide Ligase in DMF***
Liuqin Zhu and Wu Yukie
Institute of Biophysics, Chinese Academy of Sciences, China
Yang Yonghua and Yang Shengli
Shanghai Research Center of Biotechnology, CAS, China

B - 10 ***At Play in the Fields of Molecular Biology: Antibody Affinity Maturation via Combinatorial Genetics***
Jennifer Anne Maynard, Brent L. Iverson and George Georgiou
Department of Chemical Engineering, University of Texas at Austin

B - 11 ***Protein Engineering of a Lytic b-1,3-Glucanase Enzyme able to Permabilize the Yeast Cell Wall***
B.A. Andrews, A. Olivera, M.Casas, O. Salazar, J. Molitor and J.A. Asenjo
Center for Chemical Engineering, Department of Chemical Engineering, University of Chile, Chile

B - 12 ***Aqueous Two-Phase Processes for the Recovery of Aroma Compounds Produced by Micelial***

Cultures
Marco Rito-Palomares and Alejandro Negrete
Centro de Biotecnología-ITESM, Mexico
Leobardo Serrano and Enrique Galindo
Instituto de Biotecnología-UNAM, Mexico

B - 13 ***Peptide Affinity Chromatography of Human Clotting Factor VIII: Column Experiments with Peptides Derived From the Factor VIII-Binding von Willebrand Factor Region***
Karin Amatschek, Rainer Hahn, Roman Necina and Alois Jungbauer
Institute for Applied Microbiology, Austria
Djuro Josic
Octapharma, Vienna

B - 14 ***Selection of Optimum Affinity Tags for Immobilized Metal Affinity Chromatography Using a Phage Display Peptide Library***
Richard Pasquinelli, Ya Chen, Richard R. Koepsel, Rex Shepherd and Mohammad Ataai
Department of Chemical Engineering & Center for Biotechnology & Bioengineering, University of Pittsburgh, USA

B - 15 ***Human Blood Clotting Factor VIII-Binding Peptides Derived from a Combinatorial Peptide Library: Screening, Characterization and Application for Affinity Chromatography of Recombinant and Plasma Derived FVIII***
Alois Jungbauer, Karin Amatschek, Roman Necina and Eva Schallaun
Institute of Applied Microbiology, University of Agriculture,
Forestry & Biotechnology, Austria
Horst Schwinn and Djuro Josic
Octapharma Pharmazeutika Produktionsges.m.b.H., Austria

B - 16 ***Microscale Gradients of Biomolecules for Cell Adhesion and Neurite Extension***
D.N. Adams, C.L. Hypolite, E.Y. Kao and W.-S. Hu
Department of Chemical Engineering and Materials Science, University of Minnesota, USA
M.D. Distefano
Department of Chemistry, University of Minnesota, USA
P.C. Letourneau
Department of Cell Biology and Neuroanatomy, University of Minnesota, USA

B - 17 ***Development of a System for Advanced Life Support in Space Based on a Loop of Bioreactors***
F. Gòdia, J. Albiol, J.L. Montesinos, A. Vernerey, J. Pérez, F. Cabello, N. Creus, A. Morist and X. Mengual
Departament d'Enginyeria Química, Universitat Autònoma de Barcelona, Spain
C. Lasseur
European Space Research and Technology Center, European Space Agency, The Netherlands

B - 18 ***Cysteine Mutations and the Cellular Redox Environment are Critical for the in vivo Folding and Assembly of the Non-Disulfide Bonded P22 Tailspike Protein***
Anne Skaja Robinson
University of Delaware

B - 19 ***Effect of Arginine on the Oxidative Renaturation of Hen Egg-White Lysozyme***

Lars Waldmann, Rainer Rudolph and Eliana De Bernardez Clark
Department of Chemical Engineering, Tufts University and the Institute for Biotechnology, University of Halle, Germany

B - 20 ***Antemortem Diagnosis of Transmissible Spongiform Encephalopathy***
Kelvin H. Lee
Cornell University

B - 21 ***Screening of Large Cell Populations for 'Enzyme Evolution'***
Amihay Freeman
Tel Aviv University

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RELATED PROCEEDINGS APPENDIX

None.